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(71) Applicant: THEREXSYS LIMITED [GB/GB]; The Science Park, University of Keele, Keele, Staffordshire ST5 5SP (GB).

(72) Inventors: THATCHER, David, Robert; 6 Juniper Rise, Macclesfield SK10 4XT (GB). CRAIG, Roger, Kingdon; Jubilee House Farm, Spen Green, Smallwood, Sandbach, Cheshire CW11 0XA (GB). WILKS, Paula, Elizabeth; 5 Arrowsmith Drive, Alsager, Stoke on Trent ST7 2JK (GB). CUNLIFFE, Vincent, Trevor; 21 Queen Street, Knutsford WA16 6HZ (GB). WELSH, John, Hamilton; 4 Melville Court, Clayton, Newcastle under Lyme ST5 4HJ (GB).

(74) Agent: HALLYBONE, Huw, George; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

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(57) Abstract

The invention is based on the discovery of a synthetic virus like particle containing a plurality of peptides capable of condensing nucleic acid and condensed nucleic acid. The plurality of peptides has a low polydispersion index and each peptide of said plurailty is a heteropeptide. The nucleic acid may encode sequences of therapeutic benefit. The synthetic virus like particle is self-assembling and may be designed so as to be capable of targeting a particular cell or tissue type and delivering nucleic acid to be incorporated into the chromosomal or extrachromosomal sequences of the target cells or tissues.

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IMPROVED PHARMACEUTICAL COMPOSITIONS FOR GENE THERAPY

FIELD OF THE INVENTION

The present invention relates to pharmaceutical compositions useful in the treatment of diseases by gene therapy, and in particular to compositions which rely on nucleic acid condensation agents.

BACKGROUND TO THE INVENTION

Gene therapy relies on efficient delivery of DNA to target cells, and expression of the delivered DNA in the nucleus of such cells. Different modes of DNA delivery have been proposed, and these involve both viral and non-viral delivery of gene sequences.

Early experiments on introducing DNA into mammalian cells in vitro utilized DNA in precipitated form with low efficiency of transfection and required selectable marker genes (Wigler et al. (1977) Cell 16, 777-85; Graham and Van der Eb (1979) Proc. Natl. Acad. Sci. USA 77 ,1373-76 and (1973) Virology 52, 456)). Since this time molecular biologists have developed many other more efficient techniques for introducing DNA into cells, such as electroporation, complexation with asbestos, polybrene, DEAE, Dextran, liposomes, lipopolyamines, polyornithine, particle bombardment and direct microinjection (reviewed by Kucherlapati and Skoultchi (1984) Crit. Rev. Biochem. 16, 349-79; Keown et al. (1990) Methods Enzymol. 185, 527). Many of these methods are unsuitable for use clinically since they give highly variable and relatively poor levels of transfection. Another obstacle to the wider use of existing transfection

complexes resides in their instability in vivo. It has been shown that particles of a similar size to the transfection complexes of the prior art are rapidly and efficiently removed from the blood by the reticuloendothelial system (Poste and Kirsch, Bio/Technology 1, 869 (1984)).

Loyter and Volsky (Cell Sur. Rev. 8, 215-266 (1982)) and Kaneda et al. (Exp. Cell Res. 173, 56-69 (1987)) describe the reconstitution of viral envelopes as biological carriers including carriers of DNA. In this approach, naturally occurring viruses are isolated, dissolved in detergent containing solvents, the viral nucleic acid removed and the remaining viral components reconstituted in the presence of plasmid DNA. However, this technology has proven to be extremely expensive and difficult to scale up. Moreover, serious safety concerns are connected with the pharmaceutical use of extracted viruses.

Other non-viral gene delivery systems described in the literature merely extend observations on transfection using DNA condensed using synthetic polymers. For example, soluble DNA/polylysine complexes can be generated (Li et al., Biochem. J. 12, 1763 (1973)). Polylysine complexes tagged with asialoglycoprotein have been used to target DNA to hepatocytes in vitro (Wu and Wu, J. Biol. Chem. 262, 4429 (1987); U.S. Patent 5,166,320). Lactosylated polylysine (Midoux et al. (1993) Nuc. Acids Res. 21, 871-878) and galactosylated histones (Chen et al. (1994) Human Gene Therapy 5, 429-435) have been used to target plasmid DNA to cells bearing lectin receptors, and insulin conjugated to polylysine (Rosenkrantz et al. (1992) Exp. Cell Res. 199, 323-329) to cells bearing insulin receptors. However, Wagner et al. (*supra*) have shown that the latter approach is even less efficient than standard methods of transfection, and may therefore be considered unsuitable for pharmaceutical-development. Monoclonal antibodies have been used to target DNA to particular cell types (Machy et al. (1988) Proc. Natl. Acad. Sci. USA 85, 8027-8031; Trubetskoy et al. (1992) Bioconjugate Chem. 3, 323-27 and WO 91/17773 and WO 92/19287).

Peptides derived from the amino acid sequences of viral envelope proteins have been used in gene transfer when coadministered with polylysine DNA complexes (Plank et al. (1994) J. Biol. Chem. 269, 12918-24). Trubetskoy et al. (supra) and Mack et al. ((1994) Am. J. Med. Sci. 307, 138-143) suggest that cocondensation of polylysine conjugates with cationic lipids can lead to improvement in gene transfer efficiency. WO 95/02698 discloses the use of viral components to attempt to increase the efficiency of cationic lipid gene transfer.

Disulfide bonds have been used to link the peptidic components of a delivery vehicle

(Cotten et al. (1992) Meth. Enzymol. 217, 618-644); see also, Trubetskoy et al. (supra) However, the chemical modification of the various components, although group specific, is not regio-specific and leads to enormous molecular heterogeneity of the conjugated product. Disulfide bonds are also known to be unstable in biological fluids and thus limits the potency of such compounds in practice.

Similar heterogeneity is also produced by other standard conjugation methods such as carbodiimide coupling through side chain carboxyl groups (Wu et al. (1991) J. Biol. Chem. 266, 14338-42). However, in addition to the above disadvantages, the resulting amide bond coupling the components is chemically stable within the cytosol and makes the components difficult to separate.

More specific coupling chemistry has been employed by Cotten et al. (supra). This method involves oxidation of the carbohydrate moieties using periodate, followed by subsequent reaction with polylysine. The Schiff base so formed was reduced with sodium cyanoborohydride to form a stable amide bond. However, due to the large number of available lysine residues, the resulting amide bond was linked at random to the polylysine component.

Trubstskoy (supra) observed increased efficiency of a conjugate made up of a heterogeneous polylysine moiety linked through the N-terminus non-specifically to amino functions on a monoclonal antibody.

Many prior art methods employ highly heterogeneous components linked by conjugation chemistry which itself leads to more heterogeneity. This heterogeneity leads to poor control during preparation and large batch-to-batch variability, low potency and poor solution stability.

Scale up and reproducible manufacture of the gene delivery vehicles described in the literature are problematic because of the extreme heterogeneity of the products and components of those systems. Key parameters such as quality control, process control and product identification are thus rendered imprecise. Therefore, an object of the invention is the development of a reproducible and scalable production process for pharmaceutical compositions which facilitate delivery of exogenous DNA to a target cell with high efficiency.

Another object of the invention is to provide an improved nucleic acid delivery vehicle that is capable of evading the reticuloendothelial system and thus may withstand degradation in vivo.

Another object of the invention is to provide an improved gene delivery complex having chemical components of defined stoichiometry and therefore reduced heterogeneity.

Another object of the invention is to provide gene therapy products having improved properties as pharmaceutical compositions with improved therapeutic benefit.

Yet another object of the invention is to provide a nucleic acid delivery complex that may be designed to target a selected cell population or to target a broad range of cell types.

Yet another object of the invention is to provide a nucleic acid delivery complex that has an exceedingly low level of non-specific cell targeting.

Yet another object of the invention is to provide a self-assembling virus-like particle, using defined stoichiometry of components.

Yet another object of the invention is to provide pharmaceutical formulations for gene delivery which exhibit increased transfection efficiency.

SUMMARY OF THE INVENTION

The invention encompasses a synthetic virus like particle (i.e., a transfection complex or gene delivery vehicle) for transfecting nucleic acid into a mammalian cell, the synthetic virus like particle comprising a recombinant nucleic acid, a plurality of nucleic acid condensing peptides, the peptides being non-covalently associated with the recombinant nucleic acid such that the nucleic acid is in condensed form,wherein each nucleic acid condensing peptide is a heteropeptide, and the plurality of nucleic acid condensing peptides has low polydispersion.

In preferred embodiments, the plural nucleic acid condensing peptides comprise a first nucleic acid condensing peptide and a second nucleic acid condensing peptide, wherein the first nucleic acid condensing peptide comprises a first functional group covalently bound thereto. The first nucleic acid condensing peptide may further comprise a second functional group which may be directly bound to the peptide or may be covalently bound to the first functional group, where the first funcitonal group is bound to the peptide. Alternatively, a second nucleic acid condensing peptide also may comprise a second functional group covalently bound thereto, the second functional group being different from the first functional group. The first and second nucleic acid condensing peptides may have identical or different amino acid sequences.

The functional groups which are bound to peptides useful in the invention include a ligand that is an antigenic peptide or a ligand that targets a specific cell-type such as a monoclonal antibody, insulin, transferrin, asialoglycoprotein, or a sugar. The ligand thus may target cells in a non-specific manner or in a specific manner that is restricted with respect to cell type.

The functional group also may comprise a lipid such as palmitoyl, leyl, stearoyl or cholesterol.

The functional group also may comprise a neutral hydrophilic polymer such as polyethylene glycol (PEG), or polyvinylpyrrolidine (PVP).

The functional group also may comprise a fusogenic peptide such as the HA peptide of influenza virus.

The functional group also may comprise an enzyme such as a recombinase or an integrase.

The functional group also may comprise an intracellular trafficking protein such as a nuclear localization sequence (NLS).

In a particular preferred embodiment, that is, wherein the second functional group is covalently linked to a first funtional group which is linked directly to the peptide, the first functional group may comprise one of a lipid or a neutral hydrophilic polymer such as PEG and the second functional group a ligand that targets a cell surface receptor. For example, when the first functional group comprises a lipid, the second functional group may comprise a ligand that targets a cellular receptor. When the first functional group comprises PEG, the second functional group may comprise a ligand that targets a cellular receptor. The ligand may be, for example, one of a sugar moiety or a ligand whose cellular receptor is restricted to a cell-type, and thus the target cell population may be unrestricted or restricted as to cell type. Alternatively, when the first functional group comprises a lipid, the second functional group may comprise PEG.

The first nucleic acid condensing peptide may comprise 8-24 positively charged amino acid side groups, more preferably the number of positively charged amino acid side groups is 12-18.

Preferably, the ratio of positive/negative charges in a synthetic virus like particle that is capable of targeting a specific mammalian cell type is within the range 0.5-3 per phosphate residue in the nucleic acid; this ratio more preferably being within the range 0.8 - 1.2.

Preferably, the ratio of positive/negative charges in a synthetic virus like particle that is unrestricted with respect to the type of cell it targets is in within the range of 0.5 - 5 per phosphate residue in the nucleic acid, and more preferably within the range of 1.2 - 2.

The invention also encompasses a plurality of nucleic acid condensing peptides which are used to formulate a synthetic virus like particle of the invention, wherein each nucleic acid condensing peptide of the plurality being a heteropeptide, and the plurality of nucleic acid condensing peptides having low polydispersion, the peptides being further characterized in that,

when contacted with recombinant nucleic acid, the peptides are able to non-covalently associate with the nucleic acid to form a synthetic virus like particle containing condensed recombinant nucleic acid, and the synthetic virus like particle being characterized in that, when contacted with a mammalian cell, the particle can transfect nucleic acid into the cell.

The plurality of nucleic acid condensing peptides may comprise a first nucleic acid condensing peptide and a second nucleic acid condensing peptide, wherein the first nucleic acid condensing peptide comprises a first functional group covalently bound thereto, and the second nucleic acid condensing peptide may comprise a second functional group covalently bound thereto, the second functional group being different from the first functional group.

A preferred nucleic acid condensing peptide comprises an amino acid sequence of the generic formula

$$NH_2\text{-}A\text{-}(X_1X_2Y_1Y_2)_nX_3X_4\text{-}(Z_1Z_2Z_3Z_4)\text{-}(X_5X_6Y_3Y_4)_mX_7X_8BCOOH$$

wherein each of X_{1-8} is, independently, an amino acid having a positively charged group on the side chain; wherein each of Y_{1-4} is, independently, a naturally occurring amino acid which promotes alpha helix formation; wherein each of Z_{1-4} is, independently, a naturally occurring amino acid with at least 3 amino acids having a high propensity to form a stabilized turn structure; wherein A is an amino-terminal serine or threonine residue; wherein B is any amino acid; and wherein n = 2 - 4 and m = 2.

Other preferred peptides are those wherein each of X_{1-8} is, independently, lysine, arginine, 2.4-diamino-butyric acid or ornithine; wherein each of Y_{1-4} is, independently, glutamic acid, alanine, leucine, methionine, glutamine, tryptophan or histidine; wherein each of Z_{1-4} is, independently, asparagine, glycine, proline, serine, and aspartic acid; wherein B is any one of alanine, glutamic acid or cysteine.

It is also contemplated according to the invention that peptides useful in the invention may contain one or more internal Serine, Threonine, or Cysteine residues, preferably at a position in the sequence which will be exposed for conjugation to a selected ligand, and thus not on the positively charged (nucleic acid oriented) face of the α-helix. This positioning of selected reactive amino acid residues within the peptide and oriented such that they do not contact the face of the peptide that contacts nucleic acid permits conjugation of the peptide with other functional peptides by bonds of selected and defined stability. Cysteine allows specific conjugation via the thiol side chain to compounds containing other reactive thiol groups (via disulfides), alkylating

functions (to form thioether bonds), or other thiol reactive groups such as maleimide derivatives. Bonds of "defined stability" are described hereinbelow, and include bonds such as acid labile bonds (hydrazone) or linkages that are less stable in the reducing environment of the cytosol (disulfide). Such bonds are useful for carrying functional groups on the synthetic virus like particle.

Preferred peptides which fall within this generic sequence include:

NBC7 TRRAWRRAKRRAARRCGVSARRAARRAWRRE-OH; and

NBC11 H-TKKAWKKAEKKAAKKCGVSAKKAAKKAWKKA-NH_2

Thus, a nucleic acid condensing peptide according to the invention may contain: 1) helix-forming amino acids, 2) a repeating three-dimensional structure that contacts the major groove of the nucleic acid, 3) suitable chromophores for quantitation, and 4) a number of "handles" (i.e., reactive sites) for regio-specific conjugation of ligands which form accessory functional domains.

Nucleic acid condensing peptides of the invention also may include portions of H1 (sequence I, II or III below) which are identified herein as sequences which possess the ability to condense nucleic acid. Therefore, a nucleic acid condensing peptide of the invention can comprise a linear combination of the following three consensus sequences where the total sequence length is >17 residues:

Sequence I:

-K-K-X-P-K-K-Y-Z-B-P-A-J-

where: K is Lysine, P is Proline; A is Alanine; X is Serine, Threonine or Proline; Y is Alanine or Valine; Z is Alanine, Theonine or Proline; B is Lysine, Alanine, Threonine or Valine; and J is Alanine or Valine.

Sequence II

-X-K-S-P-A-K-A-K-A-

where: X is Alanine or Valine; K is Lysine; S is Serine; P is Proline; and A is Alanine.

Sequence III

-X-Y-V-K-P-K-A-A-K-Z-K-B-

where: X is Lysine or Arginine; Y is Alanine or Threonine; Z is Proline, Alanine or Serine; B is Lysine, Threonine or Valine; K is Lysine; P is Proline; A is Alanine.

A preferred peptide is NBC1, which has the following structure:

NH_2-[SV40 NLS]-[Seq I]-[Seq II]-[Seq III]-[SV40 NLS]-[Seq I]-C-COOH,

where -C- is Cysteine;where the SV40 NLS has the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Gln; and sequence

H-PKKKRKVEKKSPKKAKKPAAKSPAKAKAKAVKPKAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-OH.

Another preferred nucleic acid condensing peptide of the invention will have an amino acid sequence that falls within the following generic sequence:

NH_2-X-$(Y)_n$-C-COOH

where X is either absent or Serine or Threonine; Y is sequence I, II or III as defined above; n is 2-6; and C is Cysteine.

Particularly preferred peptides according to the invention are the following:

NBC2 has the structure: NH_2-[Seq III]-[SV40 NLS1]-[Seq I]-C-COOH,

where -C- is Cysteine.

NBC8 has the structure: NH_2-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine.

NBC13 has the structure: NH_2-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine.

NBC10 has the structure: NH_2-[Seq I]-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine; the amino acid sequences of which are as follows:

NBC2 H-KAVKPKAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-OH;

NBC8 H-KKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-OH;

NBC13 H-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-OH;

NBC10 H-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-OH

The invention also features a synthetic virus like particle for transfecting nucleic acid into a mammalian cell, the synthetic virus like particle comprising a recombinant nucleic acid, a first plurality of first nucleic acid condensing peptides, each peptide comprising a covalently linked first functional group, a second plurality of second nucleic acid condensing peptides, wherein each nucleic acid condensing peptide is a heteropeptide and each plurality of nucleic acid condensing peptides has low polydispersion, wherein each plurality of nucleic acid condensing peptides is non-covalently associated with the recombinant nucleic acid such that the nucleic acid is in

inventi n with a neutral hydrophilic polymer, as described herein.

The invention also encompasses a pharmaceutical formulation for administering a recombinant nucleic acid to a patient, comprising a synthetic virus like particle of the invention in admixture with a pharmaceutically acceptable carrier.

The pharmaceutical formulation may further comprise an endosomal disruption agent, or a neutral hydrophilic polymer.

The invention also encompasses a method of introducing a recombinant nucleic acid into a patient, the method comprising administering to the patient a therapeutically effective amount of a synthetic virus like particle of the invention or a pharmaceutical formulation of the invention.

The invention also encompasses a method of making a synthetic virus like particle for transfecting mammalian cells with a recombinant nucleic acid, the method comprising mixing a recombinant nucleic acid with a plurality of nucleic acid condensing peptides under conditions sufficient to permit formation of a synthetic virus like particle containing condensed nucleic acid, wherein the nucleic acid condensing peptide is a heteropeptide and the plurality of nucleic acid condensing peptides has low polydispersion.

Preferably, the mixing step comprises mixing the nucleic acid with a plurality of nucleic acid condensing peptides wherein the plurality comprises a first nucleic acid condensing peptide and a second nucleic acid condensing peptide, each nucleic acid condensing peptide being a heteropeptide and each plurality of nucleic acid condensing peptides having low polydispersion, wherein the first nucleic acid condensing peptide comprises a first functional group covalently bound thereto.

The invention also encompasses a method of making a synthetic virus like particle for transfecting mammalian cells with a recombinant nucleic acid, the method comprising mixing a recombinant nucleic acid with a first plurality of first nucleic acid condensing peptides and a second plurality of second nucleic acid condensing peptides under conditions sufficient to permit formation of a synthetic virus like particle containing condensed nucleic acid, wherein each first and second nucleic acid condensing peptide is a heteropeptide and each first and second plurality of nucleic acid condensing peptides has low polydispersion.

The method also may include the step of mixing the synthetic virus like particle with an endosomal disruption agent.

The first nucleic acid condensing peptide may comprise a first functional group covalently

linked thereto, and the second nucleic acid condensing peptide may comprise a second functional group covalently linked thereto. The first and the second nucleic acid condensing peptides may have the same or different amino acid sequences. The first and second functional groups also may be the same or different groups.

Each nucleic acid condensing peptide may have additional functional groups bound thereto, and these functional groups may be directly bound to the peptide or indirectly bound to the peptide through another functional group.

The mixing step of this method of the invention may comprise the step of selecting a ratio of first and second functional groups such that the proportions of first and second nucleic acid condensing peptides having covalently bound thereto first and second functional groups, respectively, that are mixed with recombinant nucleic acid correspond to this ratio.

The invention also encompasses high precision chemistry in that the bonding position on a nucleic acid condensing peptide may be selected for covalent linkage to a functional group, and the position on the functional group for bonding to an amino acid on a nucleic acid condensing peptide also may be selected. The selection of a bonding position on a nucleic acid condensing peptide may include, for example, selecting the amino acid position on the first nucleic acid condensing peptide for covalent linkage to the first functional group and selecting the amino acid position on the second nucleic acid condensing peptide for covalent linkage to the second functional group. The bonding position may be an amino or carboxy terminal amino acid position of the nucleic acid condensing peptide.

The invention also encompasses a method of making a synthetic virus like particle for transfecting mammalian cells with a recombinant nucleic acid, the method comprising a) contacting a plurality of first nucleic acid condensing peptides with a recombinant nucleic acid in high salt concentration for a time sufficient to allow noncovalent association of the plurality of nucleic acid condensing peptides with the nucleic acid and condensation of the nucleic acid, b) diluting the salt concentration of step a) to a lower salt concentration and bringing the solution to a concentration of neutral hydrophilic polymer which permits stable particle formation.

Preferably, the method also includes an additional step, after or coincident with step b), of adding a plurality of second nucleic acid condensing peptides, each second peptide comprising a lipid group covalently linked thereto, wherein each first and second nucleic acid condensing peptide is a heteropeptide and each plurality has low polydispersion.

Preferably, in step b), the dilution is performed simultaneously with the adding of the plurality of second nucleic acid condensing peptides. Alternatively, the dilution is performed prior to the adding of the plurality of second nucleic acid condensing peptides.

The invention also encompasses a method of making a synthetic virus like particle for transfecting mammalian cells with a recombinant nucleic acid, the method comprising a) forming a mixture of a plurality of first nucleic acid condensing peptides and a recombinant nucleic acid in high salt concentration, b) incubating the mixture of step a) for time sufficient to allow noncovalent association of the plurality of nucleic acid condensing peptides with the nucleic acid and condensation of nucleic acid, and c) contacting the mixture of step b) with a plurality f second nucleic acid condensing peptides comprising a lipid group covalently linked to a second peptide, wherein each first and second nucleic acid condensing peptide is a heteropeptide and each plurality of nucleic acid condensing peptides has low polydispersion.

Further features and advantages of the invention will become more fully apparent in the following description of the embodiments and drawings thereof, and from the claims.

DESCRIPTION OF THE FIGURES

Before describing the preferred embodiments off the invention, the drawings will be discussed.

Fig. 1(a) is a schematic representation of a synthetic virus like particle according to the invention (10) containing a plurality of a heteropeptide (20a) and condensed nucleic acid (50);

Fig. 1(b) is a schematic representation of a virus-like particle according to the invention (11) containing a plurality of a heteropeptide having a first amino acid sequence (20a), a plurality of a heteropeptide having a second amino acid sequence (20b), a plurality of a heteropeptide (21) having a functional group (30) linked thereto, and condensed nucleic acid (50);

Fig. 1(c) is a schematic representation of a virus-like particle according to the invention (12) containing a plurality of a heteropeptide (20), a plurality of a heteropeptide (21) having a first functional group (30) linked thereto, a plurality of a heteropeptide having a second functional group (31) linked thereto, and condensed nucleic acid (50), the first and second functional groups being present in the particle in a selected ratio;

Fig. 1(d) is a schematic representation of a virus-like particle according to the invention (13) containing a plurality of a heteropeptide (20), a plurality of a heteropeptide (23) having both

a first (30) and a second (31) functional group linked thereto, and condensed nucleic acid (50);

Fig. 1(e) is a schematic representation of a virus-like particle according to the invention (14) containing a plurality of a heteropeptide (20), a plurality of a heteropeptide having a first functional group (30) linked thereto and a second functional group (31) linked to the first functional group, and condensed nucleic acid (50); and

Fig. 1(f) is a schematic representation of a virus-like particle according to the invention (f) containing a plurality of a heteropeptide (20), a plurality of a heteropeptide (21) having a first functional group (30) linked thereto, a plurality of a heteropeptide (22) having a second functional group (31) linked thereto, a plurality of a heteropeptide (25) having a third functional group (32) linked thereto, and condensed nucleic acid (50), the first, second, and third funtional groups being present in the particle in a selected ratio.

Fig. 2(a)-(h) are representative electrospray data on the mass distribution of peptides used in the invention. Each Figure shows the deconvoluted electrospray mass spectrum. Data collected on a VG Instruments Quattro II Instrument fitted with a Quadropole analyzer. Peptides were dispensed and diluted into acetonitrite: methyoxyethanol; trifluoroacetic acid and injected directly into the instrument source;

Fig. 2(a) refers to NBC1;

Fig. 2(b) to NBC2;

Fig. 2(c) to NBC4;

Fig. 2(d) to NBC7;

Fig. 2(e) to NBC8;

Fig. 2(f) to NBC9;

Fig. 2(g) to NBC10; and

Fig. 2(h) to NBC13.

Fig. 3 is a gel retardation assay for nucleic acid condensing peptides. Complexes were assembled and electrophoresis carried out as described in Example 4.1.

Fig. 4(a) is a graph showing the effect of excess nucleic acid condensing peptide on untargeted transfection by synthetic virus like particles of the invention.

Fig. 4(b) is a graph showing the effect of excess nucleic acid condensing peptide n transfection efficiency.

Fig. 4(c) is a chart showing the effect of excess nucleic acid condensing peptide on

transfection efficiency.

Fig. 5 shows the relative transfection potency of individual NBC peptides alone. The assay was performed as follows. Complexes were assembled as described in Example 4.1 using 0.6M sodium chloride, 25mM sodium phosphate buffer ph7.4; and 2μg of peptide per μg f pRSV Luc plasmid DNA. The assay performed as described in Example 4.2.1 using Jurkat Cells ($1x10^6$ cells/point) and 2.5 μg DNA per point.

Fig. 6(a) shows the relative transfection efficiency of individual NBC peptides in combination with a fixed amount of Lip 13 in the complex. Experimental details are as described for Fig. 5 except each complex was assembled in the presence of 0.15μg Lip 13 per μg DNA. NBC peptide concentrations were 2 μg peptide /1 μg DNA.

Fig. 6(b) is the same experiment as described in Fig. 6(a) except the ratio of Lip 13 was increased to 0.6 μg peptide/ 1 μg DNA.

Fig. 7 is a UV absorbance trace of the elution pattern obtained during the ion-exchange chromatography of anti-CD7-NBC2.

Fig. 8 is a UV absorbance trace of the elution pattern obtained during the ion-exchange chromatography of anti-CD33-NBC2 linked via an acid labile bond and a disulfide linkage.

Fig. 9 is a histogram showing reporter gene expression in human myeloid cells (K562 cells) treated with a plasmid containing the reporter gene condensed with anti-CD33-NBC1. The experiment was carried out by the methods described in Examples 4.1 and 4.2.1. The amount f DNA (in a complex) per assay point ($1x10^6$ cells/point) was varied from 0.15-5 μg.

Fig. 10 is a histogram showing the effect of charge ratio on transfection efficiency. Data points are the average of two replicates.

Fig. 11 is a histogram showing the effect of antibody conjugate (anti-CD7-NBC1) concentration in the nucleic acid condensing on transfection efficiency. Each data point is the average of three replicates.

Fig. 12 shows the relative transfection efficiency of targeted complexes (anti CD7/Jurkat cells) assembled with various proportions of ligand-NBC conjugate in the complex. The point of 100% condensation of RSV Luc DNA by anti-CD7-NBC1 conjugate and unconjugated NBC1 peptides was determined by separate gel retardation analyses. Complexes were then assembled as described in Example 4.1 with various proportions of anti-CD7-NBC1. Conjugated and unconjugated NBC1 were added in relative proportions so as to achieve 100% condensation f

the nucleic acid, as described in detail in Example 6.

Fig. 13 shows the time course for expression of the luciferase reporter gene after transfection of Jurkat cells with anti-CD7-NBC1 complexes assembled as described in Example 4.1 in the presence of 0.6M sodium chloride, 2.5 mM HEPES buffer, pH 7.4.

Fig. 14 shows the targeting of luciferase reporter gene DNA to HepG2 cells using insulin receptor-mediated gene transfer.

Fig. 15 shows that the level of insulin receptor targeted gene transfer is diminished by transfection in the presence of unconjugated ligand. The control delivery system in this experiment was a non-targeted gene complex composed of 2μg NBC13 per μg DNA in combination with 0.6 μg Lip8 per μg DNA.

Fig. 16 shows the transfection of HepG2 cells using mannosylated NBC1/pRSV Luc DNA complexes. Complexes were assembled as described in Example 4.1 and assayed as described in Example 4.2.2 except cells were incubated for both 24 and 90h after transfection.

Fig. 17 is a histogram showing increased transfection efficiency obtained if N-palmityl-NBC1 is incorporated in the complex. Anti CD7-NBC1 conjugate was co-condensed with increasing proportions of Lip2 as described in Example 4.1 and in the legend to Fig. 12 and used to transfect Jurkat cells as described in Example 4.2.1.

Fig. 18 is a histogram showing increased transfection efficiency obtained if N-palmityl-NBC1 is incorporated in the complex. Anti CD33-NBC1 conjugate was co-condensed with increasing proportions of Lip2 as described in Example 4.1 and in the legend to Fig. 12 and used to transfect K562 cells as described in Example 4.2.1.

Fig. 19 is a graph showing the effect of the presence of lipidated nucleic acid condensing peptide on the transfection efficiency of synthetic virus like particles. Complexes were assembled as described in Example 4.1 using NBC2 and Lip2 and assayed using HepG2 cells as described in Example 4.2.2.

Fig. 20 shows the effect of Lip 2, Lip 7, Lip 8, and Lip 13 in combination with NBC peptides NBC2, NBC7, NBC8 and NBC13 on non-targeted transfection of Jurkat cells using RSV Luc luciferase reporter gene DNA.

Fig. 21 shows the effect of Lip 13 and palmityl poly-lysine in combination with poly-lysine or NBC13 on transfection of Jurkat cells using RSVLuc luciferase reporter gene DNA.

Fig. 22 is a graph showing transfection of HepG2 and K562 cells using a synthetic virus

like particle.

Fig. 23 is a graph showing plasma stability of synthetic virus like particles containing a lipidated peptide.

Fig. 24 is a chart showing the effect of varying the concentrations of PEG and salt in a synthetic virus like particle formulation on transfection efficiency.

Fig. 25 is a chart showing the effect of varying the concentrations of DNA and salt in a synthetic virus like particle formulation containing 2% PEG on transfection efficiency.

Fig. 26(a) shows the relative transfection efficiency of formulations of RSVLuc reporter gene DNA. Duplicate 1 shows the effect of the NBC2/Lip2 complex formulated in 0.8M sodium chloride, 25mM HEPES pH 7.4. 2.5μg / assay point were diluted directly into $1x10^6$ Jurkat cells during the transfection procedure described in Example 4.2.1. Duplicate 2 shows the transfection efficiency if the complex is first diluted into 0.15M sodium chloride and 25mM HEPES. Duplicate 3 shows the transfection efficiency after dilution into the same buffer except containing 10% PEG 10,000. Duplicate 5 shows the effect observed when the complexes were assembled in the presence of 0.8M sodium chloride and 3.5% PEG 10,000 and then diluted as described for Duplicate 3.

Fig. 26(b) shows the effect of pre-assembling NBC2 complexes in 0.6M sodium chloride 25mM sodium phosphate and diluting this solution with phosphate buffered saline, phosphate buffered saline containing 0.6μg Lip2/μg DNA, into phosphate buffered saline containing 10% PEG 10,000 and into phosphate buffered saline containing 10% PEG 1000 and 0.6μg Lip 2/μg DNA.

Fig. 27 shows the effect of maintaining various formulations at either 4° C or -20° C. The formulations S6 - S19 are summarized in Example 13.

Fig. 28 is a histogram showing delivery of the synthetic virus like particle to peripheral blood mononuclear cells.

Fig. 29 is a histogram showing delivery of the synthetic virus like particle to Jurkat cells and peripheral blood mononuclear cells.

Fig. 30 is a series of photographs of cross-sections of tumor tissue which has been transfected in vivo by a synthetic virus like particle in which transfection by a lacZ reporter gene is indicated as a blue color. The experiments were performed with NBC2/Lip2 complexes assembled and formulated in a high salt formulation as described in Example 12.1.1.

Fig. 31(a)-(f) is a series of photographs f cross-sections of tumor tissue which has been transfected in vivo by a synthetic virus like particle in which transfection by a lacZ reporter gene is indicated as a blue color. The experiments were performed with NBC13/Lip2 complexes assembled and formulated in an isotonic formulation as described in Example 12.2.2.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the discovery of a highly efficient synthetic virus-like particle which, when contacted with a mammalian cell, transfers nucleic acid into the cell. The synthetic virus like particle includes a plurality of nucleic acid condensing peptides and condensed nucleic acid. The characteristics of these components of the synthetic virus like particle, and how to make and use synthetic virus like particles of the invention are described in detail below.

A nucleic acid useful according to the invention may be any form of nucleic acid, e.g., circular, linear, double-stranded, and DNA or RNA. The nucleic acid may be of any length or sequence, e.g., a 10-50 base oligonucleotide or a longer vector DNA, e.g., 1-20kb.

Nucleic acid condensing Peptides According to the Invention

Nucleic acid condensing peptides useful in the invention possess the following characteristics.

1) Nucleic acid condensing peptides useful in the invention are characterized in that the peptides are heteropeptides.

As used herein, a "heteropeptide" refers to a peptide having a selected amino acid sequence. The term "heteropeptide" refers to a peptide having an amino acid composition of at least two different amino acids. This is in contrast to a "homopeptide" in which the amino acid composition consists of identical amino acids. Thus, a homopeptide consists of 100% of the same amino acid, whereas a heteropeptide consists of a polypeptide claim in which as few as a single amino acid differs from the remainder of the amino acids in a chain, or for example 5%, 10%, 20%, 30%, 50%, 70%, 80%, 90%, etc., of the amino acids in a chain differ from the remaining amino acids in the chain. A heteropeptide is therefore a linear peptide consisting of, in terms of its selected amino acid sequence, a variety of (i.e., at least two, at least three, at least four, etc.,) types of amino acids.

As used herein, an "amino acid" refers to any of the twenty natural common amino acids, and also may refer to natural, uncommon amino acids or amino acid derivatives or analogs. An

amino acid in D- r L- form may be present in a peptide according to the invention.

2) Nucleic acid condensing peptides of the invention are also characterized in that a plurality of nucleic acid condensing peptides, or a preparation of nucleic acid condensing peptides useful in the invention has a low polydispersion index.

Nucleic acid condensing peptide preparations of the prior art, e.g., known as polycations, have a polydispersion index >1.1. This is evident in the description of polylysine peptide preparations in the prior art as having a "mean Mr" (Wu et al., 1987, J. Biol. Chem. 262;4429; Wu et al., 1988, J. Biol. Chem. 263:14621; and Wu et al., 1988, Biochem. 27:887) or having "an average chain length" (Wagner et al., 1991, Proc. Nat. Aca. Sci. 88:4255; Wagner et al., 1992, Proc. Nat. Aca. Sci. 89:7934) or an "average degree of polymerization" (Birnsteil et al., EPO532525). Polylysine preparations having a mean Mr or an average chain length are used in the prior art to condense DNA for gene delivery. Such preparations are disclosed as having an average degree of polymerization of a given number of lysine groups, e.g., "polylysine 90" has an average degree of polymerization of 90 lysine groups; "polylysine 190" has an average degree of polymerization of 190 lysine groups; etc (Birnsteil et al., supra.) Preparations of polylysine are available commercially from Sigma Corporation and are known by one of skill in the art and documented upon purchase to have an actual distribution of sizes within each sample which varies per sample, and may vary, for example, from 30% - 150% of the material being distributed. Thus, the actual peptide length within such a sample may be, for example, 80% above or 80% below the stated average length of the peptide. The length of the peptide is thus reported as an average of various sizes which average is determined by low-angle light scattering analysis of individual lots of chemically synthesized peptide.

Nucleic acid condensing peptide preparations useful according to the invention have a low polydispersion index (PDI), also termed index of polydispersity. As used herein, a "low" polydispersion index refers to a PDI of <1.01. Nucleic acid condensing peptides useful in the invention have a PDI <1.01, and preferably have a PDI <1.001. Nucleic acid condensing peptide preparations of the invention, of course, may have a PDI of 1.0 and thus are termed "monodispersed". As used herein, a "high" polydispersion index refers to a PDI of >1.1, >1.2, >1.3, and generally in the range of 1.1-2.0.

The polydispersion index is used to characterize the molecular weight distribution of polymeric compounds. The PDI for a polymer having a homogeneous distribution of molecular

weights (i.e., where the polymer preparation contains a uniform molecular weight) is 1.0, and for a highly heterogeneous polymer preparation (i.e., where the polymer preparation contains a wide distribution of molecular weights) the value approaches 2.0.

The polydispersion index of a peptide preparation may be determined according to two methods which are described below: 1) using light scattering and colligative properties of the peptide preparation to determine the weight and number average molecular weights of the peptides in a preparation; or 2) using electro-spray mass spectrometry to determine the molecular weights of peptides in a given preparation. It is critical to the invention that the PDI be calculated according to the most accurate of the two methods, i.e., electro-spray mass spectrometry. That is, the former method of calculating the PDI provides only a rough estimate of the PDI in that, for a given peptide preparation, the ratio of Weight Average Molecular Weight / Number Average Molecular Weight for heterogeneous polymers may be determined, respectively, by light scattering and from colligative properties of the peptide preparation (G. Odian in Principles of Polymerisation, John Wiley and Sons, 1981). One such colligative property is viscosity (*supra*). However, these parameters for measuring weight and number average molecular weights, are not accurate enough to be used in the determination of the PDI of peptides having low polydispersion, because they provide only a rough estimate of the PDI, i.e., only to within 1% of the mass of each peptide component of the preparation.

For peptide preparations having a PDI <1.01, i.e., those peptide preparations synthesized according to methods described herein, a highly accurate measurement of peptide length in a peptide preparation must be provided such that the accuracy is within 0.01% of the mass of each peptide component of the preparation, and preferably within 0.001%.

The PDI for polymer peptides disclosed herein may be calculated from analysis of the peptides by electro-spray mass spectrometry. This method gives the exact mass of each component to within 0.001%. Representative electrospray mass sysectometry data of the peptides used in the invention are provided in Fig. 2(a)-(h). The PDI values of the peptide preparations useful in the present invention are in the range of 1.0 - 1.01. Calculation of the PDI from electospray data is illustrated in Example 3. Peptide preparations which are especially useful in the invention possess a PDI <1.01, and even <1.001. In contrast, the PDI values for the nucleic acid condensing peptide preparations reported in the prior art are >1.1.

3) Amino acid length and composition of nucleic acid condensing peptides of the

inventi n.

A nucleic acid condensing peptide suitable according to the invention is a basic peptide, i.e., a peptide with net positive charge, for example a peptide or polypeptide comprising 2-50, preferably 12-40, and more preferably 15-38 D or L amino acid residues. Preferably, the polypeptide includes at least 30%, more preferably 50%, and could include as much as 80-90% basic amino acids, such as lysine, arginine, hisidine, ornithine or a non-natural amino acid containing a side chain having a secondary or tertiary amine group.

A nucleic acid condensing peptide according to the invention is preferably a peptide of, for example, 8-50 residues in length which includes a cysteine and/or threonine and/or serine residue which is available for regio-specific conjugation to a ligand, as defined herein. It is preferred that the cysteine and threonine residues be located N- and C-terminally, respectively, such that they may act as handles for covalent attachment of ligands.

Although the invention contemplates the use of heteropeptides having overall net positive charge, nucleic acid condensing peptides useful in the invention also include peptides wherein a large proportion (e.g, 30%-90%) of the amino acid composition of the peptide is a single basic amino acid species, such as lysine. For example, a heteropeptide which includes a sequence of from approximately 8 to approximately 30 lysyl residues is useful according to the invention. However, a peptide which is a homopolymer of lysyl residues is not useful according to the invention because homopolymeric polylysine tends to be cytotoxic and to stick nonspecifically to cell surfaces under certain conditions. Thus, the heteropolymeric nucleic acid condensing peptides contemplated herein are advantageous over homopolymers such as polylysine.

4) A preferred nucleic acid condensing peptide is a peptide or polypeptide having an α-helical conformation structure.

Such peptides, of which NBC7 and NBC11 (described herein) are specific examples, have been designed to interact with DNA by interacting through a conformational structure that is alpha helical. Where a functional group is covalently linked to the peptide, it follows that the functional group of the conjugate may be positioned around the turn of the alpha helical structure so as to be optimally exposed to the outer surface of the virus like particle.

We have focussed on the conformational structure of DNA-conjugate complexes (i.e., DNA noncovalently associated with a conjugate which includes a peptide of the invention covalently linked to a functional group). The relevant intermolecular interactions are refined by

building a rigid scaffold which will specifically dock with DNA and allow positioning of the functional moiety such that steric hindrance will not occur. Such a rigid scaffold is provided by any peptide which forms a stable α-helix.

A number of proteins that specifically interact with DNA (e.g., P22 cro, repressor, trp repressor, Cap, etc.) interact through an α-helix structure (10 residue) which lays across the major groove of the DNA helix. This α-helix is usually followed in the polypeptide by a reverse turn forming the helix-turn-helix motif which is characteristic of these proteins. Most of the interactions that these sequences make with the DNA are weak hydrogen bonds to the phosphate and bases. This is because the function of these domains is to recognize a particular base sequence and then trigger a conformational change. We hypothesized that if such helices are designed so that the hydrogen bonds were replaced by ionic interactions, the binding would be much stronger and useful to us as a docking/condensation peptide.

A peptide having an α-helical scaffold is designed as follows.

Using a molecular model of a 10 residue α-helix, a helical wheel and a model of B-DNA, it is apparent that strong interactions can be made using a repeating sequence motif of X_1X_2-BB-X_1X_2-BB-X_1X_2 , where X_1 is a basic residue interacting with the phosphate on one side of the major groove and X_2 is a basic residue interacting with a phosphate diametrically opposite the other side of the major groove. Either of X_1 and X_2 may be, for example, Lysine, arginine, or Histidine. Lysine, Arginine, and Histidine are preferred because both amino acids 1) are of sufficient size t bridge the major groove gap, 2) will interact strongly with phosphate and 3) are strong helix formers. If the residues BB are also residues with strong helix-forming propensities then the peptide will form a stable α-helix in solution.

Preferred nucleic acid condensing peptides according to this aspect of the invention will fall within the generic formula

NH_2-A-$(XXYY)_n$XX-($Z_1Z_2Z_3Z_4$)-$(XXYY)_m$-XX-B-COOH

where X is a naturally occurring or synthetic amino acid carrying a positively charged group on the side chain such as lysine, arginine, 2.4-diamino-butyric acid, histidine, and ornithine or a non-natural amino acid containing a side chain having a secondary or tertiary amine group;

where Y is naturally occurring amino acid which has a high propensity to promote alpha helix formation as defined by Wilt and Thornton (1988) J. Mol. Biol 203. 2221-232 such as Glutamic acid, Alanine, Leucine, Methionine or Glutamine, Tryptophan or Histidine;

where Z_{1-4} are naturally occurring amino acids with at least 3 members of the sequence having a high propensity to form stabilized turn structures as defined by Wilt Thornton (*loc. cit.*) such as, Asparagine, Glycine, Proline, Serine, and Aspartic Acid;

where A is N-terminal Serine or Threonine allowing specific oxidation of the side chain to an aldehyde group with periodic acid and thereby permitting conjugation of the peptide to another molecule carrying a reactive hydrazide or aminooxyacetyl function;

where B is any amino acid but preferably Alanine, Glutamic Acid or Cysteine; and where n = 2 - 4 and m = 2.

It is also contemplated according to the invention that peptides useful in the invention may contain one or more internal Serine, Threonine, or Cysteine residues, preferably at a position in the sequence which will be exposed for conjugation to a selected ligand, and thus not on the positively charged (DNA oriented) face of the α-helix. This positioning of selected reactive amino acid residues within the peptide and oriented such that they do not contact the face of the peptide that contacts DNA permits conjugation of the peptide with other functional peptides by bonds of selected and defined stability. Cysteine allows specific conjugation via the thiol side chain to compounds containing other reactive thiol groups (via disulfides), alkylating functions (to form thioether bonds), or other thiol reactive groups such as maleimide derivatives. Bonds of "defined stability" are described hereinbelow, and include bonds such as acid labile bonds (hydrazone) or linkages that are less stable in the reducing environment of the cytosol (disulfide). Such bonds are useful for carrying functional groups on the synthetic virus like particle.

In this aspect of the invention, a peptide will contain: 1) helix-forming amino acids, 2) a repeating three-dimensional structure that contacts the major groove of the DNA, 3) suitable chromophores for quantitation, and 4) a number of "handles" (i.e., reactive sites) for regio-specific conjugation of ligands which form accessory functional domains. Examples of such peptides include NBC7 and NBC11. In these peptides, the threonine at position 1 is available for oxidation to glyoxal and therefore for conjugation via an oxime bond, tryptophan at positions 5 and 28 are chromophores which will allow quantitation in the presence and absence of DNA. The lysine at position 9 of NBC7 and the glutamic acid at position 9 of NBC-11 are available for regio-specific conjugation via reductive amination, peptide bonds, etc., and the cysteine at position 16 for conjugation via thioether and disulfide bonds. The C-terminal glutamic acid of NBC7 is available for modification to a hydrazide and therefore for coupling via an oxime or hydrazone bond.

5) Another preferred nucleic acid condensing peptide is a peptide comprising a sequence which is derived from a sequence of histone H1 protein and other human histones.

Histone H1 is a highly basic protein of the histone family that is found in all higher organisms. Histone H1 unlike the majority of other members of this family does not assemble as an integral part of the nucleosome. Histone H1 is believed to interact primarily with those stretches of chromosomal DNA linking the nucleosome complexes of chromatin (Allen et al. Nature 288(1980). 675). Histone H1 binds to naked DNA with the same salt dependence as H1 depleted chromatin (Kumar and Walker (1980) Nucleic Acids Research 8, 3135).

Histone H1 protein is not useful as a component of a gene therapy delivery vehicle because it is not readily available and is a biological repressor of its own transcription. Recombinant histone H1 cannot readily be produced by recombinant methods, and the protein is too large to be synthesized chemically. Purification of H1 from mammalian sources other than human cells poses a safety hazard. The use of histone H1 to promote the condensation of plasmid DNA for transfection is likely to reduce expression of the delivered gene because H1 is part of a general repressor mechanism where the presence of excess histone H1 leads to reduced transcription (Weintraub, H.[1985] cell, 705-711; Croston et al. [1991] Science 251, 643).

Nucleic acid condensing peptides of the invention, however, may include those portions of H1 (sequence I, II or III below) which are identified herein as sequences which possess the ability to condense nucleic acid. Therefore, a nucleic acid condensing peptide of the invention can comprise a linear combination of the following three consensus sequences where the total sequence length is >17 residues:

Sequence I:

-K-K-X-P-K-K-Y-Z-B-P-A-J-

where: K is Lysine, P is Proline; A is Alanine; X is Serine, Threonine or Proline; Y is Alanine, Proline, or Valine; Z is Alanine, Threonine, Lysine, or Proline; B is Lysine, Alanine, Threonine or Valine; and J is Alanine or Valine.

Sequence II

-X-K-S-P-A-K-A-K-A-

where: X is Alanine or Valine; K is Lysine; S is Serine; P is Proline; and A is Alanine.

Sequence III

-X-Y-V-K-P-K-A-A-K-Z-K-B-

where: X is Lysine or Arginine; Y is Alanine, Valine, or Threonine; Z is Proline, Alanine or Serine; B is Alanine, Lysine, Threonine or Valine; K is Lysine; P is Proline; A is Alanine.

Sequence IV is a consensus sequence from all human histone sequences:

Sequence IV

-A-B-C-D-E-F-G-H-I-J-K-

where: A is preferably Lysine or Threonine; B is preferably Glycine or Glutamine; C is preferably Glycine, but can also be Aspartate, Glutamate, or Serine; D is preferably Glycine, but can also be Lysine, Valine, Glutamine, or Threonine; E is preferably Lysine or Alanine; F is preferably Alanine or Lysine, G is preferably Arginine, but can also be Valine or Isoleucine; H is preferably Alanine, but can also be Threonine, Histidine, or Proline; I is preferably Lysine, Arginine, or Glutamine; J is Alanine or Anginine; and K is preferably Lysine or Glutamine. A preferred consensus sequence is:

-K-G-G-G-K-A-R-A-K-A-K-.

NBC1 has the following structure:

NH_2-[SV40 NLS]-[Seq I]-[Seq II]-[Seq III]-[SV40 NLS]-[Seq I]-C-COOH

where -C- is Cysteine;where the SV40 NLS has the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Gln-(Dingwall and Laskey (1991) Trends Biochem. Sci. 16, 478).

Therefore, another preferred nucleic acid condensing peptide of the invention will have an amino acid sequence that falls within the following generic sequence:

NH_2-X-$(Y)_n$-C-COOH

where X is either absent or Serine or Threonine; Y is sequence I, II, III or IV as defined above; n is 2-6; and C is Cysteine.

Particularly preferred peptides are the following:

NBC2 has the structure: NH_2-[Seq III]-[SV40 NLS1]-[Seq I]-C-COOH,

where -C- is Cysteine.

NBC8 has the structure: NH_2-[Con Seq I]-[Con Seq I]-C-COOH

where -C- is Cysteine.

NBC13 has the structure: NH_2-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine.

NBC10 has the structure: NH_2-[Seq I]-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine.

Synthetic virus like particles prepared with NBC1 or NBC2 peptides are insoluble without salt at >10 µg/ml; therefore, these peptides are preferred where salt is present during particle formulation. Generally, if the particles are conformationally pure and the peptide/DNA interactions stable, then this salt effect is not observed (charged particles tend to repel each other). However, it is preferred according to the invention, as described in detail hereinbelow, that some salt be present during formulation of a synthetic virus like particle of the invention.

Sequences NBC8 - 10 are derived from part of NBC2 but lack the nuclear localization sequence and have a repeat motif (seq. I, II or III above) which has enabled us to look at the effect of peptide length on function. NBC8 has a double repeat of this sequence, NBC13 a triple repeat and in NBC10 the sequence is quadrupled.

The sequences of NBC1-10 are as follows.

NBC1 NH2-PKKKRKVEKKSPKKAKKPAAKSPAKAKAKAVKPK-AAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-COOH

NBC2 NH2-KPKAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-COOH

NBC4 NH2-KKAKSPAKAKAKAVKPKAAKPKKSPKKAKKPAYAC(Acm)-COOH

NBC5 NH2-KPKAAKPKKEVKRKKKPKKSPKKAKKPAAC(Acm)-COOH

NBC6 H2-KAKAKAKPKAKAKAKPKAKAKAKPKAKAKAKPKAKAC(Acm)-COOH

NBC7 NH2-TRRAWRRAKRRAARRCGVSARRAARRAWRRE-COOH

NBC8 NH2-KKSPKKAKKPAAKKSPKKAKKPAYC(Acm)-COOH

NBC9 H2-TKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-COOH

NBC10 NH2-KKSPKKAKKPAAKKSPKKAKKPAAKKSP-KKAKKPAAKKSPKKAKKPAYC(Acm)-COOH

NBC11 NH2-TKKAWKKAEKKAAKKCGVSAKKAAKKAWKKA-CONH2

NBC12 H2-TKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAYC(Acm)-COOH

NBC13 NH2-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAYC(Acm)-COOH

NBC14 NH2-TKKKKKKKKKKKKKKKKKKC-COOH

The syntheses of these peptides are described in detail in Examples 1 and 2. Other nucleic acid condensing peptides whose synthesis is not specifically described herein are synthesized essentially as described in the examples.

6) Nucleic acid condensing peptides of the invention may contain one or more covalently linked functional groups.

As used herein, a "functional group" refers to a protein, peptide, lipid, or chemical group that is covalently linked to a DNA binding peptide, as defined herein, and which has a biological function with respect to particle stability in biological fluids, entry into a cell, or delivery of DNA to the cell nucleus, or integration into the chromosome. The covalent linkage may be a stable or labile linkage, as defined hereinbelow. Where the functional group is a peptide, the covalent linkage may be a peptide bond, thus creating a fusion protein.

Examples of functional groups useful according to the invention include but are not limited to the following: a) a ligand, such as i) an antigenic protein or peptide, or ii) a targeting molecule having a cognate receptor on the surface of a target cell; b) a lipid; c) a neutral hydrophilic polymer; d) an endosomal disruption agent; e) an enzyme, and f) an agent which promotes intracellular trafficking into the nucleus, and combinations thereof.

a) Functional groups which are ligands.

A ligand will include an antigenic protein or peptide recognized by a cell as foreign (for example, a dendritic cell) and thus will promote uptake of the synthetic virus like particle by the cell. A ligand also may be a targeting molecule having a cognate receptor on the surface of a target cell. For example, including but not limited to antibodies; lectins; sugars such as monosacharrides or oligosacharrides, for example, mannose, galactose, fucose, and sialic acid; transferrin; and

asialoglycoprotein. For example, antibodies which target cells include but are not limited to anti-integrins for targeting keratinocytes, anti-E-selectin for targeting endothelial cells, anti-CD2, CD4 or CD8 for targeting Tcells, anti CD33 for targeting monocyte/macrophage/dendritic cell precusors, anti-HLA Class II (constant region) for targeting macrophages, Bcells and activated dendritic cells, anti-CD80, CD19 or CD22 for targeting Bcells; and antibodies for targeting cancer cells include but are not limited to anti PEM (polymorphic epithelial mu in) for colon and breast cancers, anti CEA (carcinoembryonic antigen) colorectal tumors, anti MAGE for melanomas, and anti-EGFR-! (epidermal Growth factor receptor-1) for lung and breast cancers.

A targeting ligand useful according to the invention will recognize and bind with high (i.e., Ka=<10nM) and specific affinity a specific ligand on the target cell type. In practice, the most useful targeting ligands are monoclonal antibodies or a receptor molecule, such as insulin r epidermal growth factor, or alternatively the binding domain of a receptor binding molecule such as that of E-selectin.

One type of targeting ligand useful according to the invention comprises the protein hormone insulin or a derivative of insulin to direct the synthetic virus like particle to cells expressing the insulin receptor, where the insulin or insulin derivative retains receptor binding properties when conjugated to a nucleic acid condensing peptide. The synthesis of insulin and insulin derivative conjugated peptides is described in Example 1, 6 and 7, and the use of such peptides for targeted gene transfer to insulin receptor-bearing cells is described in Example 6.

The efficiency of transfer of nucleic acid from the synthetic virus like particle to the target cell may be dependent on the density of the ligand in the synthetic virus like particle. Receptor-ligand interactions which trigger endocytosis usually involve initial oligomerization of the membrane bound receptor. Therefore, it is preferred that the synthetic virus-like particle described herein be used in an amount effective to allow for clustering of bound receptor at the cell surface. One way in which such clustering is achieved is to synthesize a peptide containing a clustered ligand, such as a clustered monoclonal antibody, as described in Example 6.

The targeting ligand can also be a sugar residue coupled directly to the amino group f an amino acid of a nucleic acid condensing peptide or indirectly linked to the peptide, for example, through a PEG functional group. Syntheses and uses of such derivatives are described in Examples 1, 6, and 11.

b) Functional groups of the invention also include lipids and thus, when conjugated to a

peptide of the invention, form lipid-derivatized nucleic acid condensing peptides.

As used herein, the term "lipid" refers to a four - thirty carbon molecule that is insoluble in water and soluble in alcohol. The term includes fats, fatty oils, essential oils, waxes, sterols, cholesterols, phospholipins, glycolipins, sulfolipins, aminolipins, chromolipins, and fatty acids. The nucleic acid condensing peptide can be specifically modified according to the invention by condensation with an lipid, for example, an activated ester of a fatty acid. The fatty acid is ideally either palmitic acid, oleic acid, such as dioleoylphosphatidylethanolamine, myristic acid, r cholesterol, although other fatty acids, such as stearic acid, may also be employed. An example of the synthesis of N-palmitoyl-NBC2 (Lip 2) is provided in Example 1 and 7, and its use in gene transfer is described in Example 7. The effect of the presence of the lipid component on transfection efficiency of the synthetic virus-like particle is dramatic (see results presented in Fig. 17-23) leading to >40-fold increase in activity. The phenomenon is unrelated to the effect observed during DNA transfection using cationic lipids, where activity is proportional to the level of cationic lipid in the complex, in which the ligand density required for transfection is orders of magnitude higher (e.g., 10x-100x) than the ligand density required for gene transfer according to the present invention.

Cholesterol refers to a lipid having the carbon ring structure shown in Example 1 and also includes derivatives having attached groups.

One advantage conferred by the presence of lipidated peptides in the particle or the particle formulation is resistance of the particle to degradation. The presence of lipidated peptide in the particle formulation confers higher resistance to inactivation by human plasma. Experiments presented in Example 7 show that the presence of lipidated peptide in the particle formulation increases the relative level of transfection after exposure of the synthetic virus like particle to various levels of human plasma in the transfection medium (Fig. 23).

c) A functional group which is a neutral hydrophilic polymer.

Little is known about the solvation and stabilization of polycation-nucleic acid complexes and most of the literature to date has been focused on the importance of electrostatic interactions and the nature and concentration of the counter ion (e.g. Garcia-Ramirez *et al* (1994) Biopolymers 34, 285-292; Mistra and Honig (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 4691). In the case of proteins, the theory of how solvation and solvent additives influence the stability of protein structures is well advanced (reviewed Arakawa *et al* (1993) 1-28; Timasheff (1992) in

Stability of Protein Pharmaceuticals Part B, pp 265-285). The theory of the action of these stabilizing additives is that stabilization is brought about by the thermodynamically unfavorable interaction of the protein and solvent additive. This tendency of the protein surface to repel the stabilizing additive from the immediate vicinity of that surface is a phenomenon called selective hydration or negative binding. That is, the concentration of this additive near the protein surface is much lower than in the bulk solvent. Thus, when proteins are denatured or unfolded, the surface area of the polypeptide that is in contact with the solvent increases enormously. Because the preferential exclusion of the solvent additive from the solvent layer in contact with the protein is clearly proportional to surface area, the native compact state will be favored over the denatured (extended) state as less solvent additive will have to be preferentially excluded from the native state. Compounds which have this effect are those which typically do not have strong polar or ionic interactions with the protein, do not have strong hydrogen bonding functionalities and have a tendency to repel non-polar molecules. Such compounds are polyols, such as sugars, glycer l and hydrophilic polymers such as polyetheylene glycol, methyl cellulose, poly vinyl alcohol, polyvinylpyrrolidine, hydroxy propyl cellulose, pullulans, polyoxamers, polyoxamines, polysorbates, and poly (2-hydroxy propyl) methacrylamide.

Much of the work in the literature on condensation of nucleic acids has focused on the influence of ionic strength on aggregation and size (Garcia Ramierez *ibid*; Ferkol, WO 95/25809). The degree of condensation achieved by mixing cations and DNA in the presence of salt generally do not approach the degree of condensation found in natural chromatin. We hypothesized that increased DNA condensation could be achieved by applying the principles of protein structural stabilization to cation-DNA interactions. That is, when multivalent cations neutralize the high negative charge density of the DNA, the forces of electrostatic repulsion which ensure the DNA helix is extended disappear and the DNA structure collapses (condensation) as hydrophobic interactions between segments of complexed helix predominate. Given the length and rigidity of the DNA helix, there will still be forces which will increase the packing density of the DNA in the collapsed state.

We hypothesized that the use of co-solvents which are excluded from the surface of hydrophobic surfaces, such as hydrophilic polymers (polyethylene glycol [PEG], pluronic polyols, polyvinyl alcohol, polyvinylpyrrolidine) should lead to higher levels of nucleic acid compaction, lower aggregation, more stable formulations, and thus smaller overall size of the particle. This

has been observed experimentally.

As used herein, the term "neutral hydrophilic polymer" includes those polmeric molecules which act as co-solvents with peptides of the invention, including polyols, such as sugars, glycerol and hydrophilic polymers such as polyetheylene glycol, methyl cellulose, poly vinyl alcohol and poly- vinylpyrrolidine, hydroxy propyl cellulose, pullulans, polyoxamers, polyoxamines, polysorbates, and poly (2-hydroxyl propyl) methacrylamide. Such polymeric molecules will fall within the molecular weight range 1000 - 100,000; preferably within the molecular weight range 1000 - 50,000; and most preferably within the range 5000 - 10,000.

A "neutral hydrophilic polymer" is used herein as in two forms. That is, a neutral hydrophilic polymer may be used according to the invention as a functional group which is, directly or indirectly, covalently linked to a peptide useful in the invention, or it may be used as a co-solvent during formulation of a synthetic virus like particle (see below). Without being bound to any one theory, it is believed that either use of a neutral hydrophilic polymer according to the invention results in better nucleic acid compaction, smaller overall size, and greater stability of the synthetic virus like particle, and thus produces better transfection efficiency of the particle with respect to target cells.

A neutral hydrophilic polymer may be linked to a peptide, and thus presented as a functional group, as described in detail in the Examples.

d) A functional group which is an endosomal disruption agent.

A functional group useful according to the invention includes a ligand which serves to promote uptake of the synthetic virus like particle by a cell. Many peptide molecules are known in the art which have the ability to promote uptake of a molecule into the cell, e.g., by disrupting membrane structure. The most useful of such peptides for synthetic virus like particles are those which undergo a pH dependent conformational change, such as the HA peptide from the influenza virus. The structure and synthesis of such a peptide is provided in Example 8.

Additional fusogenic peptides useful according to the invention are as follows. The fusogenic peptide from Sendai Virus has the amino acid sequence:
H-Phe-Phe-Gly-Ala-Val-Ile-Gly-Thr-Ile-Ala-Leu-Gly-Val-Ala-Thr-Ser-Ala-Gln-Ile-Thr-Ala-Gly-Ile-Ala-Leu-Ala-Glu-Ala-Arg-Glu-Ala-Lys-Arg-OH (D. Rapaport and Y. Shai, J. Biol. Chem. 1994,*263*,15124-15131). The fusogenic peptide sequence from HIV gp41 protein:
H-Ala-Val-Gly-Ile-Gly-Ala-Leu-Phe-Leu-Gly-Phe-Leu-Gly-Ala-Ala-Gly-Ser-Thr-Met-Gly-Ala-

Arg-Ser-OH (M. Rafalaski, J.D. Lear and W.F. DeGrado, Biochemistry 1990,*29*,7917-7922). The fusogenic peptide sequence from Paradaxin: H-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Gly-Gln-Glu-OH (D. Rapaport, G.R. Hague, Y. Pouny and Y. Shai, Biochemistry 1993,*32*,3291-3297). The fusogenic peptide sequence from Melittin: H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2 (C.R. Dawson et al., Biochem. Biophys. Acta: 1978,*510*,75).

A mechanism by which a fusogenic peptide is believed to promote uptake of a synthetic virus like particle by the cell is as follows. At neutral pH the peptides have a random structure and little or no interaction with the cell membrane. The peptides enter the endosomal compartment by diffusion or preferably by being carried in as part of the virus like particle. As the pH of the endosome drops, the HA peptide forms an α-helical structure or aggregates thereof and these insert into the endosomal membrane and disrupt its integrity.

e) A functional group according to the invention may be an enzyme.

As used herein, an enzyme is defined as a molecule which, when covalently linked to a nucleic acid condensing peptide of the invention, is capable of effecting a biological activity involving breakage and reformation of a covalent bond. Examples of such functional groups include a recombinase or an integrase, both of which promote recombination of the recombinant DNA with cellular DNA, or an intracellular trafficking enzyme, or reporter enzyme such as horseradish peroxidase, as described in Example 9.

f) A functional group according to the invention may be a nuclear localization sequence.

Nuclear localization sequences useful in the invention include sequences which resemble the short basic NLS of the SV40 T antigen described hereinabove; the bipartite NLS of nucleoplasmin; the ribonucleoprotein sequence A1, the small nuclear ribonucleoprotein sequence U1A, and human T-lymphotrophic virus-1Tax protein; the HIV matrix protein NLS; and the nuclear translocation components importin/hSRP1 and Ran/TC4. Of particular use in the invention is a nuclear localization consensus sequence KXX(K/R) flanked by Pro or Ala (Roberts (1989) Biochem. Biophys. Acta. 1008, 263, herein incorporated by reference), or the nuclear localization sequence of nucleoplasmin (Dingwall and Laskey, Trends in Biotech. 16, 478 (1991), or the NLS from antennapedia (Derossi et al., Jour. Biol. Chem. 269, 10444 (1994)).

The invention thus also encompasses a nucleic acid condensing peptide which is linked to

a non-basic nuclear localization sequence whose natural function is the transport of nucleoprotein. Such sequences are known in viruses, e.g.,influenza nucleoprotein(Davey et al. Cell 40, 667 (1985)), HIV MA protein (Gallay et al., Cell 80, 379 (1995) . Nucleoprotein transport sequences also occur in proteins which transport ribonucleoprotein complexes, e.g., hnRNP Al protein (Siomi and Dreyfuss J. Cell. Biol. 129, 551 (1995)) . The synthesis of a nucleic acid condensing peptide (NBC2) linked to the M9 nuclear localization sequence of hnRNP Al is described in Example 10.

A nuclear localization sequence may be covalently linked to a nucleic acid condensing peptide of the invention as a functional group, or it may be present within the sequence of the nucleic acid condensing protein via peptide bond linkage as a fusion peptide.

Nucleic acid condensing sequences especially useful in the invention comprise not only a nucleic acid condensing domain, but also a nuclear localization sequence which directs transport of the vehicle to the nucleus. Examples of such nucleic acid condensing peptides are NBC1 and NBC2, described herein, which include the SV40 T antigen nuclear localization sequence within the peptide sequence, as shown in Examples 1 and 2. Example 10 shows that higher transfection activity is associated with use of NBC2 as a condensing peptide over NBC5. NBC5 has the same structure as NBC2 except that the NLS has been inactivated by reversing the amino acid sequence of this part of the sequence. It is within the scope of the invention to combine peptides having different sequences, e.g., NBC2 (containing a nuclear localization sequence) and NBC9 (no nuclear localization sequence).

Linkage of Functional Group to Nucleic acid condensing Peptide.

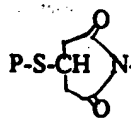
A nucleic acid condensing peptide of the invention which is conjugated to a functional group may be so conjugated via a bond which is stable enough to allow association of the peptide and the functional group to the cell surface and into the cell, if desired.

The following types of linkages are contemplated according to the invention.

1. Stable Linkages

When bond stability is not an important variable (e.g. polymerization of the basic polymer unit) then a stable linkage is preferred. Preferred stable bonds are amide, thioether and oxime.

P-C=N-0-NBC	Oxime
P-S-CH_2 -NBC	Thioether



Thioether

R'(R")C-NH-NBC Amide

P is any of the above-listed functional groups, and NBC refers to a nucleic acid condensing peptide. The synthesis of thioether linked components is provided in Example 6. Amide linkages may be formed by reduction of Schiff base bonds formed by reaction of aldehydes and amino compounds. An example of this synthesis is given in Example 6.

2. Acid Labile Linkages

After endocytosis, neither the functional group nor the fusogenic peptide need remain bound to the synthetic virus like particle. Upon entry of the particle into the cell, the newly formed endocytotic vesicle rapidly acidifies and incorporation of pH labile bonds in the structure can be used to shed these components from the particle. The preferred pH labile linkage is the unreduced hydrazone bond. Hydrazone bonds are readily synthesized by the reaction of aldehydes with substituted hydrazides.

P-CH=NNH-NBC Hydrazone

The synthesis of such structures is provided in Examples 6 and 8. The synthesis of a protease-cleavable acid labile linkage is described in Example 8.

3. Reducible Linkages

The cytosol of mammalian cells is kept under net reducing conditions by the synthesis of reduced glutathione. Disulfide bridges, therefore, are cleaved upon absorption of molecules into the cytosol. This linkage is therefore useful for the coupling of peptides to the synthetic virus like particle whose function is not necessary after the particle has entered the cell (for example, a targeting protein or a fusogenic peptide). Disulfide linkages are readily synthesized by employing thiol residues activated with the 2-pyridyl group (Carlsson et al., Biochem. J. 173, 723-737 (1978)).

P-S-S-NBC Disulfide Linkage

Examples of the synthesis of such compounds are provided in Examples 6 and 8.

Linear or Branched Chain Polymers

The synthetic virus particle may comprise either a linear or branch chain polymer described in general terms by the formulae:

LINEAR POLYMER: $[P_b]$

L

NH_2-(X_a)CONH(Y)COR^1

BRANCHED POLYMER:

NH_2-(X_a[L-P_b]$_n$CO[NH(Y)

CHCO]$_m$NHCH$_2$(Y)COR^1

NH_2-(Xa[L-P_b]$_n$CO[NH(Y)

Where:

P is a protein or peptide or other chemical substance and b is 1-20, where P belongs to one of the following groups:

(1) A targeting protein or peptide which specifically interacts with a structure on the surface of the target cell.

(2) (1) above in combination with (i.e., conjugated to) a protein, peptide or other chemical substance which facilitates the penetration of membrane structure (plasma, endosome or nuclear) or interacts with cellular components to enhance transport to the nucleus.

(3) (1) above in combination with (i.e., conjugated to) a lipid derivative with or without the agent described in (2) above.

(4) (1), (2) or (3) above in combination with a protein, peptide or other chemical substance which enhances the expression of the delivered genetic material such as an integrase or recombinase enzyme or other enzymatic function which enhancestransport of the complex to the nucleus or aids integration of DNA.

X_a is a nucleic acid binding component comprising an amino acid sequence or other biological sequence containing monomer units which are capable of binding to nucleic acid, and preferably containing at least one nuclear localization sequence, where a is 1-5;

L is a linker sequence comprising an amide, hydrazone,reduced hydrazone, disulfide, thioether or disulfide bond. The linker sequence may be a lipid or polyethylene glycol.

Y is (-CH_2)$_t$, where t is 1-6, and preferably 1-5;

R^1 is -OH, -NH_2 or O(CH_2)$_n$$CH_3$ where n = 0, 1-3,

wherein, in the branched polymer, the second chain containing a P group can be the same

as or different from the first chain.

Characteristics of Synthetic Virus Like Particles of the Invention.

1) Overall Charge

Synthetic virus like particles of the invention will possess an overall (net) charge as follows. Synthetic virus like particles which are designed so as to target a particular cell type, and therefore contain a targeting ligand, will possess an overall charge in the range of 0.5 - 3.0, more particularly in the range of 0.5 - 2.0, and optimally in the range of 0.8 - 1.2. Particles which do not target a particular cell type, but are designed so as to transfect a broad range of cell types will possess an overall charge in the range of 0.5 - 5.0, more particularly in the range of 1.0 - 3.0, and optimally in the range of 1.3 - 3.0.

The overall charge (i.e., the balance of positive and negative charge species) of the synthetic virus like particle are determined as follows. The number of moles of phosphate residues present in the nucleic acid component of the particle is estimated based on the amount of DNA to be used in the condensation reaction:

nM Phosphate in Condensation Reaction = (μg DNA/0.62) x 2

(Assumes average molecular weight of one base pair = 620.)

The number of moles of positively charged groups of each peptide is calculated based on the mass of the condensation peptides and conjugates to be added to the DNA:

nM Positively Charged Groups = (μg Peptide/Molecular Weight x 10-3) x No. of Positive Charges in Sequence.

The Charge Ratio is then:

$$= \sum (\text{nM Positively Charged Groups})_n \; / \; \text{nM Phosphate}$$

where n is each constituent peptide in the condensation reaction.

The nucleic acid condensing activity of peptides having different charges and the transfection efficiencies of synthetic virus like particles having different charge ratios are presented in Example 4. Transfection efficiencies of particles containing varying amounts of ratios of positively/negatively charged residues are presented in Example 7. Highly efficient transfection may be obtained using particles of the invention which do not contain a targeting ligand and are therefore untargeted with respect to a specific cell type. Such particles are highly efficient with respect to transfection where the ratio of positive/negative charges is greater than 1.25.

2) Overall Size

It is preferred according to the invention that the size of a virus like particle fall within the range of 5nm to 500nm. It has been found that the efficiency of uptake of the particle by a cell dramatically decreases when the particle size is greater than 500nm. This is likely due to the size of the endosomal pores in a given cell type. Particle size is measured by lasar light scattering or atomic force microscopy, or electron microscopy. Therefore, the size of a particle of the invention will vary depending upon the cell type and the size of endosomal pores in a given cell type.

3) Ratio of nucleic acid condensing peptides/nucleic acid molecules.

A synthetic virus like particle according to the invention will have a ratio of the number of peptide/the number of nucleic acid molecules in a particle that is within the range of 10/1 to 1,000,000/1. This ratio will depend upon the relative sizes of the peptide and nucleic acid molecules, the degree of condensing activity of the peptide, and the degree of condensation that the nucleic acid attains. More particularly, the range will be 100/1 -10,000/1. For example, for NBC2 in combination with an 8kb vector, a useful ratio for untargeted delivery of the vector to cells is approximately 5000:1 (relative numbers of molecules). For NBC2 conjugated to insulin in combination with an 8kb vector, a useful ratio for targeted delivery of the vector to cells is approximately 1000:1. Where the nucleic acid is an oligonucleotide of, e.g., 10-50 nucleotides in length, the ratio of peptide/oligonucleotide is in the range of 0.1-10.0 and is preferably 0.5-1.0.

Formulation of Synthetic Virus Like Particles of the Invention.

1) Particle Preparation.

A synthetic virus like particle of the invention is formulated such that the nucleic acid and the peptide preparation are prepared in equal volumes of the same buffer. The nucleic acid is agitated while the condensing peptide preparation is added at the rate of 0.1 volume per minute. The complex is left at room temperature for at least 30 minutes prior to addition to the target cells and can be stored at 4°C. The particle is centrifuged to remove any aggregated material and then assayed for gene transfer.

For use as a pharmaceutical reagent, it is preferred that the nucleic acid/peptide complex, after it is left at room temperature for 30 minutes, is filtered through a sterile 0.2μ filter, e.g., a hydrophilic nylon membrane filter. Filtration of synthetic virus-like particles described herein does

not significantly reduce yield, and in fact often results in 100% recovery of the particles.

For efficient nucleic acid transfer to a target cell, it is critical that particles of the invention contain highly condensed nucleic acid. The condensing properties of nucleic acid condensing peptides useful in the invention are disclosed in detail herein. In order to assess whether nucleic acid condensation has occurred to a degree sufficient to allow for efficient gene transfer, a gel retardation assay may be performed which assesses the ability of the peptides present in the particle to condense nucleic acid. The gel retardation assay is performed as follows.

(i) Conjugates or peptides are assayed for their ability to condense DNA using the following method:

A concentration of nucleic acid is selected, for example 20, 30, or 40 μg/ml and possibly 50, 60, 70, or 100 μg/ml, and prepared in a low salt buffer, e.g., 150 mM NaCl.

In one embodiment, the required amount of DNA is made up to 20 μg/ml in 150 mM NaCl; 25 mM HEPES, pH 7.4, or in 0.6 M NaCl; 25 mM HEPES, pH 7.4 and aliquoted between wells on a multiwell plate. The amount of conjugate or peptide required to give positive charge:phosphate ratios of between 0.1 and 5.0 is calculated. This is made up to an equal volume to the DNA aliquots (0.05-0.5 ml) in either 150 mM sodium chloride; 25 mM HEPES, pH 7.4 or 0.6 M sodium chloride; 25 mM HEPES, pH 7.4. The plate containing the DNA is placed on a plate shaker and shaken while the conjugate or peptide is added at a rate of 0.1 volume per minute. After addition of the condensing peptide is complete, the solution is incubated at room temperature for at least 30 minutes. A sample for each positive charge:phosphate ratio is subjected to electrophoresis on an agarose gel. The gel is stained with ethidium bromide and visualized under UV light. Condensed DNA remains in the well of the gel and does not migrate in the electric field.

The ability of free NBC2 peptide and monoclonal antibody-NBC peptide conjugates to condense nucleic acid is described in Example 4.

(ii) Transfection Efficiency of Synthetic Virus Like Particles of the Invention.

Synthetic virus like particles are assayed for their ability to transfer genes into peripheral blood cells. For studies aimed at determining transfection efficiency, the plasmid DNA contains a marker gene for firefly luciferase. For pharmaceutical applications, the plasmid contains a gene whose expression will have a beneficial therapeutic effect. The particle is incubated with the blood cells and the mixture may be subject to electroporation if desired. After a further incubation, the

cells are lysed and are assayed for gene expressi n. In the case of the luciferase reporter, luciferin and ATP are added to lysed cells and the light emitted is measured with a luminometer.

Cells are harvested on the day of assay by centrifugation at 1200 rpm for 5 min at room temperature. The cell pellet is resuspended in phosphate buffered saline and re-centrifuged. This operation is performed twice. The cell pellet is then suspended in RPMI 1640 (Gibco Ltd.) to make up a suspension of 2.7×10^6 cells per ml. The cells are then aliquoted into tubes and 0.75 ml of RPMI medium added, followed by 0.04-0.08 ml of 1 mM fusogenic peptide or more preferably 100 mM chloroquine and finally 0.25 ml of DNA-complex solution. The transfection is then allowed to proceed by incubating the cells at 37°C for 4 h. After this time, the cells are harvested by centrifugation at 2000 rpm. The cells are then suspended in 1 ml of RPMI and re-centrifuged. Finally, the cells are suspended in 0.5 ml RPMI containing 10% foetal bovine serum. At this stage, if necessary, the cells are electroporated at 300 V and 250 μF using conventional electroporation.

Each 0.5 ml of transfected cell suspension is transferred to a well of a 12 well plastic culture plate containing 1.5 ml of RPMI 10% FBS. The original transfection tube is rinsed with a further 1 ml of medium and the wash transferred to the culture dish making a final volume of 3 ml. The culture plate is then incubated at 37°C for 24-72 h in an atmosphere of 5% CO_2. The contents of each well in the culture dish are transferred to centrifuge tubes and the cells collected by centrifugation at 13,000 rpm. The pellet is resuspended in 0.12 ml of Lysis Buffer (100 mM sodium phosphate, pH 7.8; 8 mM $MgCl_2$, 1 mM EDTA; 1% Triton X-100 and 15% glycerol) and agitated with a pipette. The lysate is centrifuged at 13,000 rpm for 1 minute and the supernatant collected. 80 μl of the supernatant are transferred to a luminometer tube. The luciferase activity is then assayed using a Berthold Lumat L9501 luminometer. The assay buffer used is Lysis buffer containing 10 mM Luciferin and 100 mM ATP. Light produced by the luciferase is integrated over 4 sec and is described as relative light units (RLU) The data are converted to RLU/ml of lysate, RLU/cell or RLU/mg protein (protein concentration of the lysate having been determined in this case by the BioRAD Lowry assay.

Transfection efficiencies of various synthetic virus like particles of the invention into different cell lines are described in Examples 5, 6, 7, 9, 10, 13, and 15.

2) Stoichiometry of Particle Formation: Formulation of Particles Containing a Preselected Ratio of Functional Groups.

Where a synthetic virus like particle contains nucleic acid in combination with two different condensing peptide preparations, for example, a first peptide preparation having a targeting ligand conjugated thereto and a second peptide preparation having a endosomal disruption peptide conjugated thereto, the particle is formulated by incubating the three components together, at a selected ratio, for 30 minutes to 24 hours at room temperature. The ratio of the components is selected depending upon the ratio of functional groups (in this example, the ratio of targeting ligand/endosomal disruption peptide) desired in the particle. Thus, where it has been determined that a given ratio of functional groups is especially effective for transfection of a given cell type, the ratio of groups present in the virus like particle may be preselected based on stoichiometric addition to the formulation of peptides containing the functional groups.

For example, where a selected functional group is a lipid, the amount of lipid present in the particle can be varied via stoichiometric addition of a lipid-conjugated peptide to the particle formulation.

It will be appreciated according to the invention that the stoichiometry of particle formulation may be varied by combining peptide preparations wherein one or more functional groups are present on a single peptide or where a single functional group is present on a single peptide. Thus, in the former case, a peptide preparation may be added to a particle forming mixture which delivers a ratio of 1/1 of the two functional groups present on the single peptide, or which delivers a ratio of 1/1/1 or 1/2/1 (etc.) of three functional groups present on a single peptide. In addition, a second preparation may be added to the particle forming mixture which delivers a second ratio of, e.g., third/fourth/fifth functional groups to the mixture. The skilled artisan will have preselected the relative ratios of these functional groups.

Alternatively, in the latter case, where each peptide contains a single functional group, the amount of each peptide added to the particle forming mixture will determine the stoichiometry of addition of the functional group that is conjugated to the peptide.

Alternatively, the DNA may be condensed with a selected stoichiometry of condensing peptide, incubated for about 30 min, and then a second peptide containing a functional group is incubated with the condensed complex.

3) Formulation of Synthetic Virus Like Particles of the Invention with a Neutral Hydrophilic Polymer.

Synthetic Virus-like Particles of the invention may be treated with a neutral hydrophilic

polymer in order t form a more stable, smaller particle, and to increase transfection efficiency. Such polymeric molecules will fall within the molecular weight range 1000 - 100,000; preferably within the molecular weight range 1000 - 50,000; and most preferably within the range 5000 - 10,000. Such molecules are most useful according to the invention in a concentration in a given formulation within the range 0.5% - 10.0%; preferably within the range 0.5% - 5.5%; and most preferably within the range of 1% - 2%.

A neutral hydrophilic polymer is used in this aspect of the invention according to the following procedure for formulating a synthetic virus like particle.

A synthetic virus like particle is prepared by combining a selected condensing peptide with a selected amount of neutral hydrophilic polymer. If desired, a physiological (i.e., isotonic) level (e.g., 0.15M) of salt is included at this step of the formulation. Nucleic acid is then added to the mixture, and the combination allowed to incubate at room temperature for at least 1 hour. The virus like particles will have assembled during this time. However, if desired, the mixture may be incubated for as long as 24-48 hours, or stored at a cool temperature (4C) overnight.

The amount of neutral hydrophilic polymer and salt added to the mixture during particle formulation may be determined as described in Example 12, and also using guidance provided in examples 5, 7, 13 and 15, and generally as follows.

The particles may be prepared by combining 2 µg condensing peptide per µg DNA, at a DNA concentration of 100 µg/ml. Different concentrations of neutral hydrophilic polymer are chosen for testing, each at a selected salt concentration. For example, the concentrations of neutral hydrophilic polymer to be tested may be on the order of 0.5%, 1.0%, 1.5%, 2.0%, 4.0% and 5.0%, and the selected salt concentrations may be 0.2M, 0.4M, 0.6M, 0.8M and 1.0M. First, the peptide is combined with the neutral hydrophilic polymer and salt in 100 µl of 25 mM HEPES buffer pH 7.4. 10 µg nucleic aicd is added in a small volume (several µl). The particles are allowed to form at room temperature for 1 hour before being stored at 4°C overnight and used for bioassay the following day. A preferred neutral hydrophilic polymer according to the invention is PEG, at a preferred concentration of 1-2%. A preferred salt is sodium chloride at a preferred concentration of approximately 0.4M

The amount of nucleic acid and salt to be used in particle formulation may be determined as follows. Several different concentrations of nucleic acid are selected, for example, DNA concentrations (100 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml and 1000 µg/ml), each at different

salt concentrations (for example, 0.4M, 0.6M and 0.8M NaCl). Briefly, 20 µg of peptide is mixed with a neutral hydrophilic polymer, for example, 2% PEG 8000, and the selected salt concentration in 25mM HEPES pH 7.4. The concentration of peptide is such as to allow for a final peptide concentration of 200, 500, 1000, 1500, or 2000 µg/ml after addition of DNA and peptide. 10 µg DNA is then added to each mixture and mixed well. If desired, additional peptide, for example, in lipidated form, is added to the mixture. The mixtures are then left at room temperature for 1 hour before being stored at 4°C overnight. The particles are then assayed for transfection efficiency the following day.

At a salt concentration of 0.4M, the preferred nucleic acid concentration for particle assembly is 100 µg/ml or less. At higher concentrations of nucleic acid, 0.6M NaCl is optimal. The transfection efficiency of particles of the invention decreases above 500 µg/ml DNA during particle formulation.

The neutral hydrophilic polymer (e.g., Polyethylene glycol (PEG, mw range 2000-15,000)) may be present at a concentration range of 1-10% (w/v) either (1) during formation of the synthetic virus like particle or (2) after formation of the particle and during dilution of the particle into transfection medium. The efficiency of transfection was found to be significantly increased by the presence of the neutral hydrophilic polymer.

In Examples 5, 7, 12, 13 and 15, experimental results are presented showing transfection efficiencies of mammalian cells using a synthetic virus like particle formulation preparation containing a neutral hydrophilic polymer.

4) Formulation of Synthetic Virus Like Particles of the Invention in the Presence of Agents which Facilitate Endosomal Disruption.

It has been found that increased transfection efficiency is obtained where the synthetic virus like particle is formulated in the presence of endosomal disruption agents, for example, free fusogenic peptide or chloroquine.

The efficiency of transfection using a synthetic virus like particle of the invention is dramatically higher when transfection occurs in the presence of an agent which perturbs endosome function. One such potent agent is the anti-malarial drug chloroquine. The action of chloroquine can be amplified by pre-incubation of the synthetic virus like particles in a solution containing chloroquine prior to treatment of the cells (presumably by adsorption of this lipophilic molecule to the hydrophobic surface of the synthetic virus like particle).

Increased transfection efficiency is observed when synthetic virus like particles prepared in the presence of lipidated peptide are pre-incubated with chloroquine before exposure to target cells. Transfection efficiency may be further increased by increasing binding of chloroquine via elevation of the level of lipophilic substituents in the synthetic virus like particle. The range f preincubation concentrations useful according to the invention are generally from 10 μM to 70mM. At the higher dosage, the maximum amount of chloroquine administered with the synthetic virus like particle in vivo should not exceed 3.5 mg/kg body weight. For ex vivo applications, the final concentration of chloroquine after dilution from the formulation is in the range of 50-200 μM.

It has also been found that transfection efficiency is increased by extending the time period to which the target cells are exposed to the synthetic virus like particle in the presence of chloroquine. This time period may be from 2 hours to as much as 24-48 hours, with the longer incubation times resulting in increased transfection efficiency in the presence of chloroquine.

In Example 17, experiments are presented which demonstrated increased transfection efficiency using a synthetic virus like particle prepared according to a novel procedure which includes preincubation of the synthetic virus like particle with chloroquine.

5) High Salt Formulation of Synthetic Virus Like Particles of the Invention.

It has been discovered that increased transfection efficiency is obtained using a synthetic virus like particle which is prepared according to a novel procedure which includes the use of a high salt formulation. "High" salt is defined herein as being within the range of 0.5-1.0M salt; "low" salt is defined as being in the range of 0.1-0.2M salt. This procedure is as follows.

Nucleic acid is made up to 90-120 µg/ml in 25 mM HEPES buffer containing 0.6-1.0M sodium chloride. Nucleic acid condensing peptide is made up to an equivalent molarity in sodium chloride and the condensing peptide solution added to the nucleic acid solution with rapid agitation at a rate of 0.1 vol/min. The mixture is then left for at least 30 minutes at 20° C and then incubated for 1-16h at 4° C prior to dilution to a concentration of 5 µg/ml nucleic acid in RPMI medium (optionally containing albumin 1mg/ml, transferrin 50 µg/ml) and containing the endosome escape agents, fusogenic peptide (20-100 µM) or chloroquine (100-200 µM).

For particle formulation which provides particles that are stable (with respect to shelf life, as described in Example 13), and have high efficiency of transfection, as demonstrated in Example 15, it is particularly advantageous to prepare the particles by condensing the nucleic acid in high

salt (hypert nic) as described above, and then diluting this mixture in a low salt (isotonic) concentration (e.g., 0.15M) and adding a neutral hydrophilic polymer such as PEG.

6) High Salt Formulation of Synthetic Virus Like Particles of the Invention Containing Lipidated Peptides.

Transfection of mammalian cells using a synthetic virus like particle in which a lipidated nucleic acid condensing peptide is present, which particle was prepared according to a novel procedure which includes the use of a high salt formulation, as follows.

Lipidated peptides useful according to the invention cannot be incorporated in the initial high salt formulation solutions described above when the DNA concentration is above about 250-300 μg/ml. For example, when this was attempted using lipidated NBC2 (Lip2) at a DNA concentration of 400 μg/ml, the synthetic virus like particles produced exhibit minimal biological activity. Therefore, where a lipidated peptide is used in the synthetic virus like particle, the particles must be formulated such that they retain biological activity. High salt formulation of such particles is performed as follows.

Lipidated peptides are incorporated into the RPMI dilution medium at a concentration of 0-2 μg/μg DNA, (absolute concentration 0-10 μg/ml) and the solution incubated for at least 30 minutes at 37° C prior to transfection. Alternatively, the synthetic virus like particle is formulated in high salt, as described above, and the synthetic virus like particle incubated overnight at 4°C before the lipidated peptide is added to the synthetic virus like particle from stock solution of 1mg/ml lipidated peptide in 25 mM Hepes buffer containing 0.15 M sodium chloride. Transfection efficiencies of particles containing lipidated peptides, which particles are formulated in high salt, are described in Example 7.

EXEMPLIFICATION

Nucleic acid condensing peptides of the invention are synthesized so as to achieve a high degree of homogeneity in the peptide preparation, both in terms of the polydispersion index and the addition of functional groups. For example, where a peptide preparation consists of a selected amino acid sequence, care is taken during synthesis to ensure homogeneity of the heteropeptide sequence by ensuring complete coupling to the growing peptide chain. Homogeneity of the peptide preparation also is ensured by protecting potentially reactive amino acid side chains in the growing peptide.

Example 1

Nomenclature

1.1 Nucleic Acid Binding (NBC) Peptides

NBC1 NH_2-PKKKRKVEKKSPKKAKKPAAKSPAKAKAKAVKPK-AAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-COOH

NBC2 NH_2-KPKAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-COOH

NBC4 NH_2-KKAKSPAKAKAKAVKPKAAKPKKSPKKAKKPAYAC(Acm)-COOH

NBC5 NH_2-KPKAAKPKKEVKRKKKPKKSPKKAKKPAAC(Acm)-COOH

NBC6 NH_2-KAKAKAKPKAKAKAKPKAKAKAKPKAKAKAKPKAKAC(Acm)-COOH

NBC7 NH_2-TRRAWRRAKRRAARRCGVSARRAARRAWRRE-COOH

NBC8 NH_2-KKSPKKAKKPAAKKSPKKAKKPAYC(Acm)-COOH

NBC9 NH_2-TKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-COOH

NBC10 NH_2-KKSPKKAKKPAAKKSPKKAKKPAAKKSP-KKAKKPAAKKSPKKAKKPAYC(Acm)-COOH

NBC11 NH_2-TKKAWKKAEKKAAKKCGVSAKKAAKKAWKKA-$CONH_2$

NBC12 NH_2-TKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAYC(Acm)-COOH

NBC13 NH_2-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAYC(Acm)-COOH

NBC14 NH_2-TKKKKKKKKKKKKKKKKKKC-COOH

1.2 Defined Conjugates

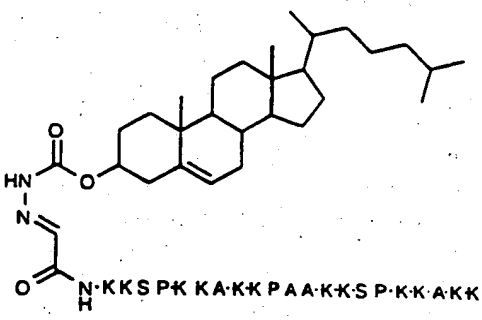
These are conjugates in which every chemical bond is a defined regio-specific bond.

1.2.1 Lipid Derivatives

N-Palmityl derivatives of the corresponding NBC peptides are called Lip1, Lip2 etc. e.g. The structure of Lip2 is:

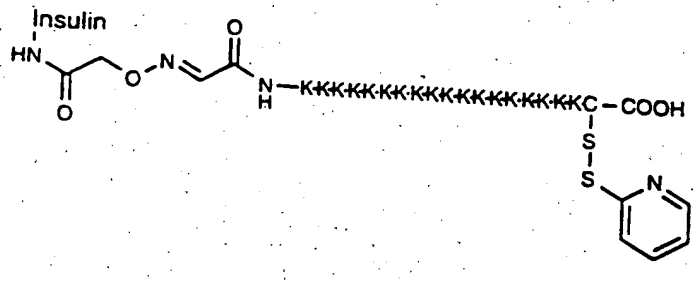
$CH_3-(CH_2)_{14}$-CONH-KPKAAKPKKPKKKRKVEKKSPKKAKK PAAC(Acm)-COOH

The Cholesteryl derivatives are named after the corresponding NBC peptides, Chol1, Chol12 etc. e.g. The structure of Chol12 (Chol^{10}-NBC12^{1} (superscripts denote the site of derivatization) is:

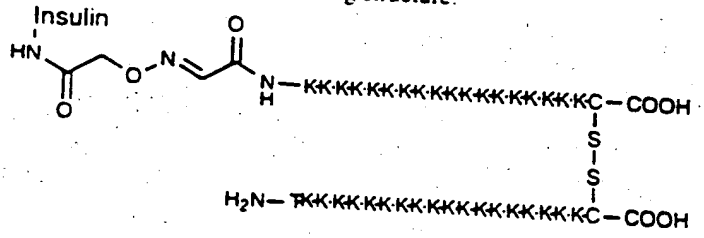


1.2.2 Insulin Conjugates

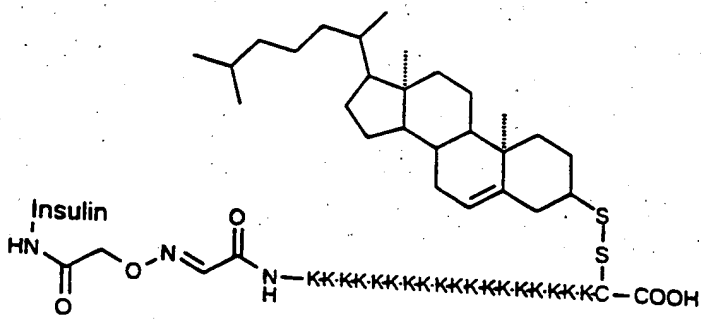
InsIa^{B1}-NBC14^{1} (superscripts refer to linkage site on ligand and peptide) has the following structure :



InsII^{B1}-NBC14^{1} has the following structure:

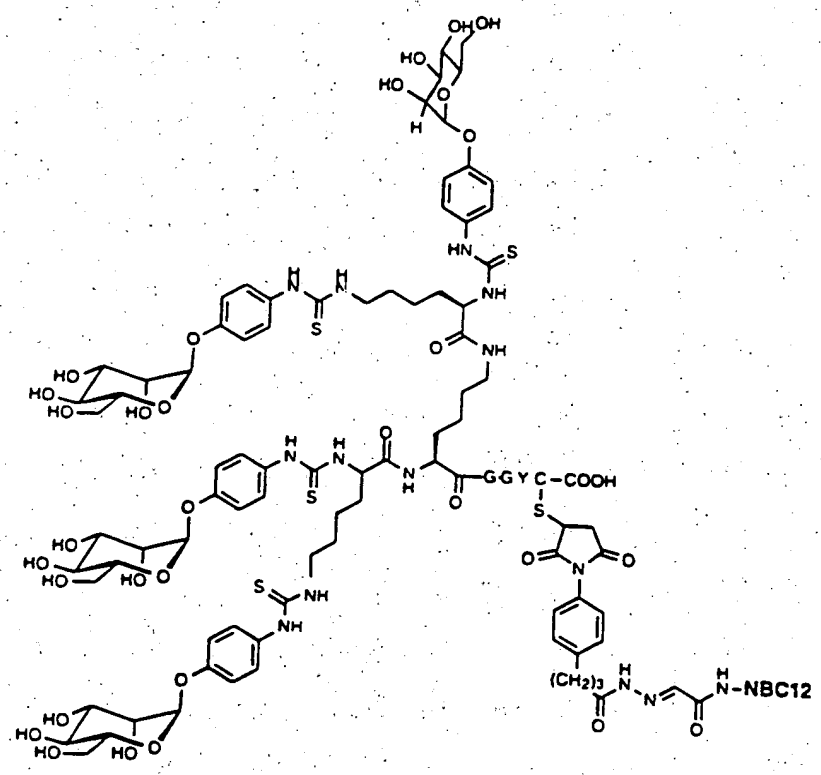


Insulin^{B1}-Chol^{B1}-NBC14^{1,18} has the following structure:

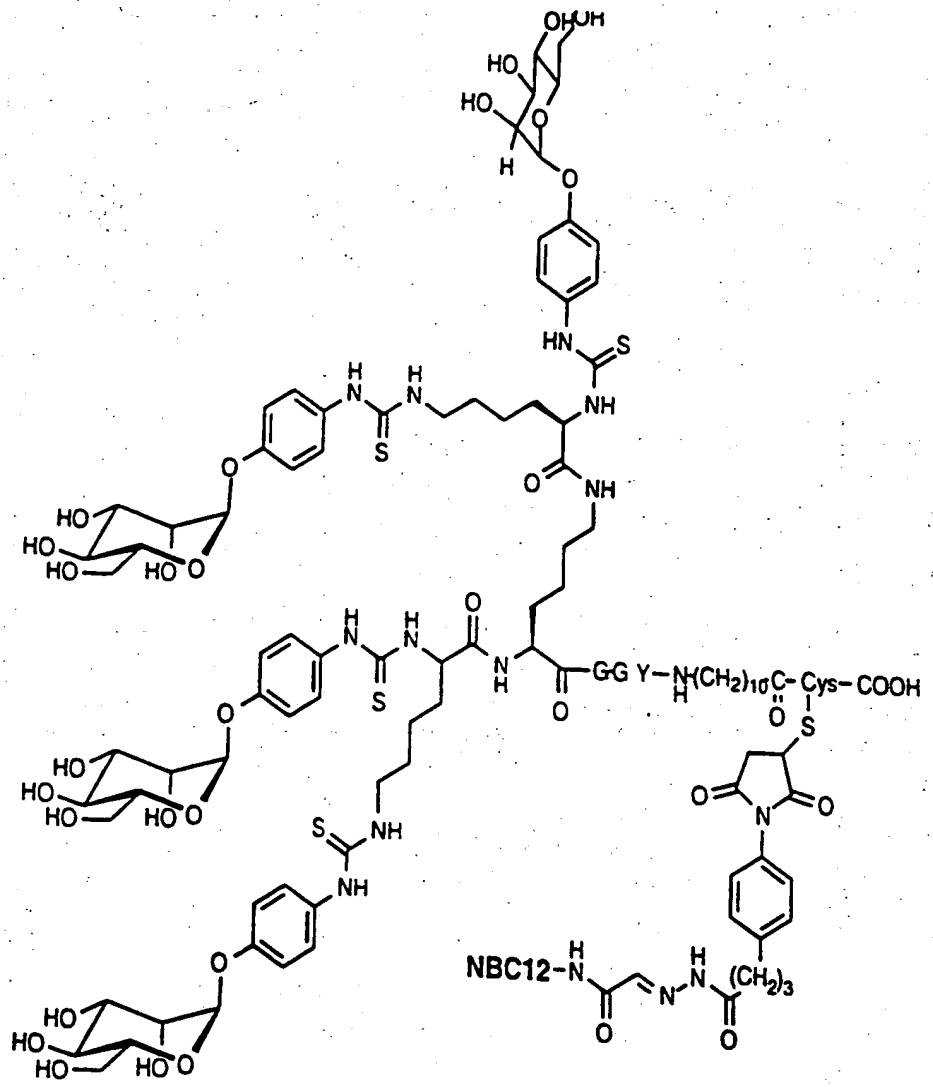


1.2.3. Glycosylated Dendrimer- Conjugates

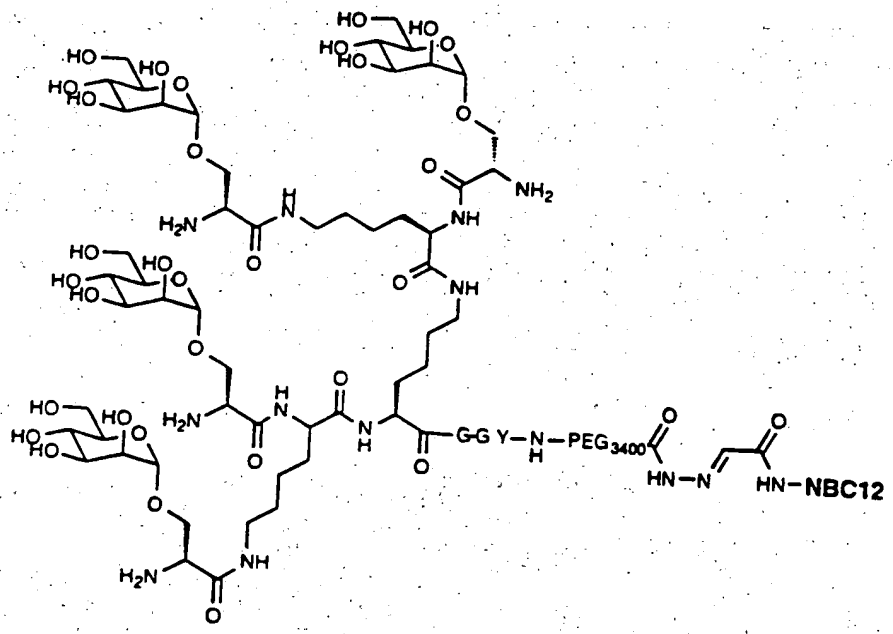
The structure of Mannosylated Lysine Dendrimer conjugated to NBC12 (Man_4Den2-NBC12[1]) is:



The structure of Mannosylated Lysine Dendrimer -undecane spacer-NBC12 (Man_4Den3-NBC12') is:

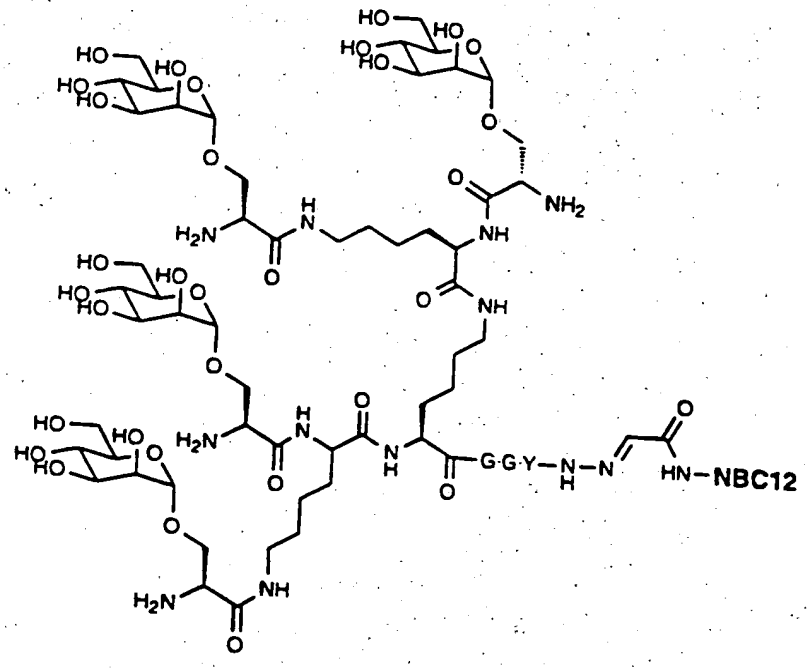


The structure of the mannosylated lysine dendrimer with a PEG spacer (Man_4Den4-NBC12) is:

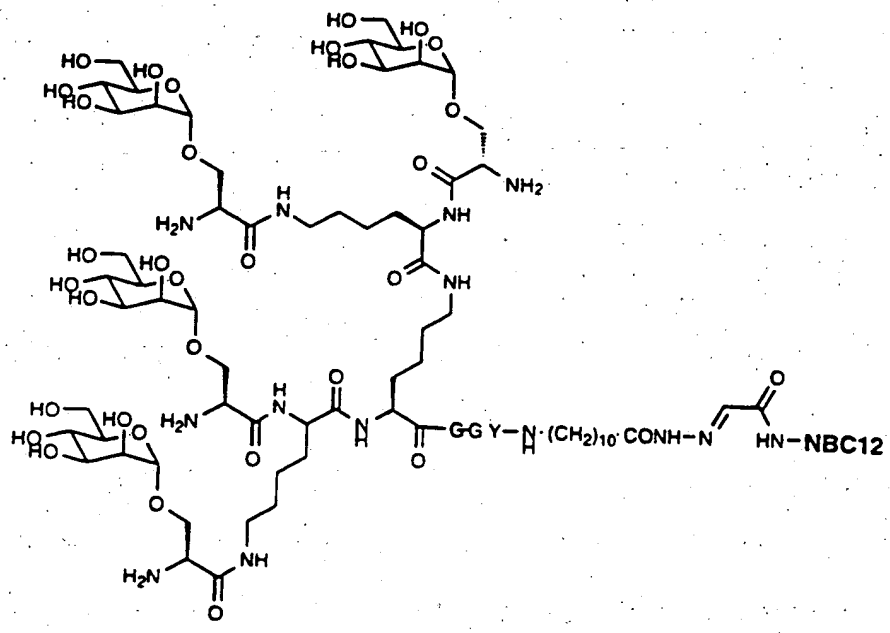


Conjugates Man_4Den5-NBC12 and Man_4Den6-NBC12 are analogues of Man_4Den2 and Man_4Den3 respectively and differ in structure by the linkage of the mannose residues to the Lysine dendrimer backbone. In these cases the sugar is linked through the hydroxyl group of N-terminal serine residues as in the case of Man_4Den5-NBC12. Also there is no cys-maleimidophenyl butyrate linker between the dendrimer and the NBC peptide.

The structure of Man4Den5-NBC12 is:



The structure of Man_4Den6-NBC12 is:



Example 2

Preparation of NBC-Peptides

Peptides NBC1-14 were synthesised using a Biosearch 9050 plus Pepsynthesizer in extended synthesis cycle mode using Fmoc-Cys(Acm)-O-PEG-PS-Resin with dimethylformamide as solvent. Deprotection of the N-terminal Fmoc-group before each coupling was achieved using a solution of 20% piperidine in dimethylformamide(1min at a high flow rate followed by 10 min at 3ml/min). The amino acids were coupled in four fold excess using O-(1H-benzotriazo-1-yl)-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole and N-ethyldiisopropylamine as activating agents. For NBC1 the coupling times started at 30 min increasing by 15mins after every 15th amino acid during the synthesis to 1.25h. for the last 15 amino acids. For all the other NBC-peptides, coupling times were set at 1hour throughout the synthesis. The following amino acid derivatives were used as appropriate for the NBC: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Glu(O'Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Ser('Bu)-OH, Fmoc-Thr('Bu)-OH, Fmoc-Tyr('Bu)-OH, Fmoc-Val-OH. After the synthesis of each peptide was complete, the N-terminal Fmoc group was removed as described above to give the free amino, side chain protected, peptide bound to the resin. Each resin was then washed into a glass vial with methanol and dried *in vacuo*.

Peptides were cleaved from the resin using a TFA/water/phenol/thioanisole/1,2-ethanedithiol (82.5: 5: 5: 5: 2.5) mixture (10ml of mixture for every gram of resin to be cleaved). The resin was then removed by filtration and washed 3 times with TFA. The combined filtrate and washings were concentrated by evaporation then precipitated using diethyl ether followed by centrifugation to give the crude peptide.

Crude peptides were dissolved in a small amount of 1% acetic acid in water and applied to a Sephadex G25 (superfine) column of an appropriate size and eluted using the same 1% Acetic acid solution. The fractions containing peptide, as determined by analytical reverse phase hplc, were pooled and lyophilised. Further purification was achieved by preparative reverse phase hplc using a Dynamax 83-221-C column and a gradient of 5-30% Acetonitrile(0.1% TFA) in Water(0.1% TFA) over 20 min. The fractions containing peptide were pooled, the acetonitrile evaporated *in vacuo* , and lyophilised.

NBC1 was then desalted by dissolving it in 0.75ml of 1% acetic acid in water and applying it to a PD-10 column which was then eluted using the same buffer. Fractions

were taken every 0.75ml and fractions 3-8 were combined and lyophilised to give the final peptide.

NBC2-NBC14 were purified by preparative rp hplc, desalted by elution on a Sephadex G25 superfine column (1.6x30cm) with 1% acetic acid in water. The resulting fractions were analysed by reverse phase hplc, pooled and lyophilised as above.

When necessary, the acetamidomethyl (Acm) thiol protecting group maybe removed using mercury (II) acetate in 30% acetic acid in water followed by precipitation of the mercury with 2-mercaptoethanol. The resulting free thiol peptide can be purified using gel filtration to give the desired product.

Example 3

Calculation of Polydispersity Index of Synthetic Peptides

The polydispersion index is used to characterize the molecular weight distribution of polymeric compounds.

$$PDI = \frac{M_w}{M_n}$$

Where M_w is the weight average molecular weight and M_n is the number average molecular weight.

The usual method for determining the weight average molecular weight of heterogeneous polymers such as poly-lysine is to use light scattering where the amount of light scattered depends on molecular size as well as the number of particles. In these cases the number average molecular weight is calculated from a colligative property of the polymer in solution such as viscosity since this property is dependent on the number of molecules of polymer per unit volume.

For example Poly-L-Lysine 25000: Sigma Product P7890, Lot No 93H-5516 has a PDI of 1.2. This result is typical for the batches of poly-lysine used in many gene transfer experiments reported in the literature.

For preparations of low polydispersity this approach cannot be used because of the relative inaccuracy of both viscosity and light scattering measurements. In these cases electrospray mass spectrometry may be used.

Fig 2 shows the deconvoluted electrospray mass spectrum of peptide NBC9 (theoretical mass 4082.2). An aqueous solution of 1mg/ ml peptide was prepared and the sample diluted in a mixture of acetonitrile:methoxyethanol:0.1% trifluoroacetic acid. The experiment was performed on a VG Instruments Quattro II instrument fitted with a

quadropol analyser. The instrument was calibrated with myoglobin and 10µl aliquots were injected directly into the instrument source.

This technique gives the exact mass and by integrating the areas of each peak the relative proportions of each component can be estimated. This enables the M_w, M_n and Polydispersion Index to be calculated.

$$M_w = \frac{\Sigma_i n_i M_i^2}{\Sigma_i n_i M_i}$$

$$M_n = \frac{\Sigma_i n_i M_i}{\Sigma_i n_i}$$

Integration of the peaks in the NBC9 spectrum gave the following relative areas:

Mass	Area (n_i)
4212	120
4084	4250
4064	420
4013	900
3838	150

From this data using the formulae above

M_w = 4070

M_n = 4067

PDI = 1.0008

Example 4

Assembly of Synthetic Virus-like Particles for the Determination of Potency *In Vitro*

4.1 Assembly and DNA Condensation

The nucleic acid is made up to 20-400 µg/ml in the buffer (usually 0.15 M to 1.0 M NaCl: 25 mM HEPES, pH 7.4). The required amount of conjugate or peptide is made up to an equal volume to the nucleic acid solution in the same buffer. The DNA is agitated moderately while the condensing agent is added at the rate of 0.1 volume per minute. The complex is left at room temperature for at least 30 minutes prior to adding to cells and can be stored at 4°C.

The following experiments were performed in order to determine the relative DNA condensing activity and transfection efficiency of nucleic acid condensing peptides disclosed herein. Nucleic acid condensing peptides which possess the characteristics of peptides of the invention are tested as described below for NBC1, 7, 8, 9, and 10; i.e., using testing parameters such as gel retardation and transfection efficiency.

Figure 3 shows the comparative gel retardation behavior of NBC1 (31 positive charged groups), NBC7 (16 positively charged groups), NBC8 (13 positively charged groups), NBC9 (19 positive charged groups) and NBC10 (25 positively charged groups). 1 µg aliquots of plasmid RSVluc DNA (20 µg/ml in 150 mM NaCl in 25 mM HEPES,pH 7.4) were aliquoted into a multi-well plate (50 µl volume). The amount of each of the peptides required to give positive charge: phosphate ratios of 0, 0.5, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5 and 4.0 was calculated and made up to 50µl in 150 mM sodium chloride; 25 mM HEPES, pH 7.4. The plate containing the DNA was placed on a plate shaker and shaken whilst the peptide solution was added to the DNA at a rate of 5 µl per minute. After addition of the condensing agent is complete, the solution was incubated at room temperature for at least 30 minutes. A sample for each positive charge: phosphate ratio is electrophoresed on a standard 1% TAE agarose gel. The gel was stained with ethidium bromide and visualized under UV light. Condensed DNA remains in the well of the gel and does not migrate in the electric field.

Each gel shows the electrophoretic mobility of the same quantity of plasmid DNA after mixing with various ratios of each peptide (equal to 0, 0.15, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5, and 4.0 equivalents of positive charge (Lysine + Arginine + α-amino) per equivalent of DNA phosphate. This data shows that all four peptides are potent DNA condensing agents with at least as good a DNA condensing activity as NBC1 and that in the related series NBC8-10, NBC8, the shorter peptide has significantly weaker condensing activity than the longer peptides NBC9 and NBC10. Although NBC7 is a weaker nucleic acid condensing peptide than the other sequences (in terms of the relative mass of peptide required to bring about condensation of a given amount of DNA), figure 15 also reveals that the peptide NBC7 brings about the abolition of ethidium bromide staining at high concentrations. The other peptides, on the other hand, although excellent condensing peptides, do not have this effect, indicating a more condensed form of DNA condensed with NBC7 which is excluding this dye from the interaction with DNA.

4.2 Determination of the Relative Potency of Gene Delivery

In studies aimed at determining transfection efficiency, the nucleic acid used is plasmid DNA contains a marker gene for firefly luciferase (pRSVLuc; de Wet J.R., Wood K.V., DeLuca M., Helsinki D.R., and Subramani S. (1987) Mol. Cell. Biol. vol. 7, pp 725-737). After incubation the cells are lysed and are assayed for gene expression. In the case of the luciferase reporter gene, luciferin and ATP are added to lysed cells and the light emitted is measured with a luminometer.

4.2.1. Assay for Transgene Expression Using Cells Grown in Suspension Culture.

Cell lines such as Jurkat and K562 grow in suspension and can be transfected and the level of transgene expression (luciferase activity) determined by the following method. Cells are harvested on the day of assay by centrifugation at 1200 rpm for 5 min at room temperature. The cell pellet is resuspended in phosphate buffered saline and re-centrifuged. This operation is performed twice. The cell pellet is then suspended in RPMI 1640 (Gibco Ltd.) to make up a suspension of 2.7×10^6 cells per ml. The cells are then aliquoted into tubes and 0.75 ml of RPMI medium added, followed by 0.04 - 0.08ml of 100mM Chloroquine and finally 0.25ml of DNA-complex solution. The transfection is then allowed to proceed by incubating the cells at 37°C for 4h. After this time the cells are harvested by centrifugation at 2000rpm. The cells are then suspended in 1ml of RPMI medium and re-centrifuged. Finally the cells are suspended in 0.5ml RPMI medium containing 10% foetal bovine serum. Each 0.5ml of transfected cell

suspensi n is transferred to a well of a 12 well plastic culture plate containing 1.5ml of RPMI 10% FBS. The original transfection tube is rinsed with a further 1ml of medium and the wash transferred to the culture dish making a final volume of 3ml. The culture plate is then incubated at 37°C for 24-90h in an atmosphere of 5% CO_2. The contents of each well in the culture dish are transferred to centrifuge tubes and the cells collected by centrifugation at 13 000rpm. The pellet is resuspended in 0.12ml of Lysis Buffer (100mM sodium phosphate, pH 7.8; 8mM magnesium chloride, 1mM EDTA; 1% Triton X-100 and 15% glycerol) and agitated with a pipette. The lysate is centrifuged at 13000 rpm for 1 minute and the supernatant collected. 80µl of the supernatant are transferred to a luminometer tube. The luciferase activity is then assayed using a Berthold Lumat L9501 luminometer. The assay buffer used is Lysis Buffer containing 10mM Luciferin and 100mM ATP. Light produced by the luciferase is integrated over 4s and is described as relative light units (RLU). The data are converted to RLU / ml of lysate, RLU/cell or RLU/mg protein (protein concentration of the lysate having been determined in this case by the BioRAD Lowry assay.

4.2.2. Assay for Transgene Expression Using Adherent Cells.

The method can be modified for adherent cells such as the hepatic carcinoma cell line HepG2. Cells were plated 24h prior to transfection in 6-well tissue culture plates at a density of 1-2 x 10^5 per well in complete medium (MEM (with Earle's salts) + 1% non-essential amino acids + 10% foetal calf serum). Transfection was carried out by aspirating the culture medium and washing the cells with phosphate buffered saline. Complex (50µg/ml DNA) is layered onto the cell monolayer in a 1ml final volume containing 2.5µg DNA and 120µM chloroquine in RPMI supplemented with human albumin (1mg/ml) and human transferrin (50µg/ml).The transfection is then allowed to proceed by incubating the cells at 37°C for 4h. after which the transfection complex was removed, the cells washed with PBS and completed medium added prior to incubation at 37°C for 24-72h in an atmosphere of 5% CO2.

The cells were then harvested using trypsin to detach the cells. After addition of medium containing serum to inactivate the trypsin, cells were pelleted by centrifugation at 13,000 rpm. The cells were then suspended in 1ml of PBS and re-centrifuged. The pellet was resuspended in 200µl of Lysis Buffer (100mM sodium phosphate, pH 7.8; 8mM MgCl2, 1mM EDTA; 1% Triton X-100;15% glycerol; 0.5mM PMSF and 1 mM Dithiothreitol) and agitated with a pipette. The lysate is centrifuged at 13,000 rpm for 1 minute and the supernatant collected. 80ml of the supernatant are transferred to a

luminometer tube. The luciferase activity is then assayed using a Berthold Lumat L9501 luminometer. The assay buffer used is Lysis buffer containing 0.01mM Luciferin and 0.04375mM ATP. Light produced by the luciferase is integrated over 4sec and is described as relative light units (RLU). The data are converted to RLU / ml of lysate, RLU/cell or RLU/mg protein (protein concentration of the lysate having been determined in this case by the BioRAD Lowry assay.

It has been found that highly efficient transfection can be obtained if the gene delivery complex is assembled such that the ratio of equivalents of positively charged residues (on the DNA condensing polymer) to negative charges (on the phosphate groups of the plasmid DNA) in the formulation is in excess of 1.25. Figure 4 shows non-receptor mediated transfection by synthetic virus like particle prepared by mixing increasing amounts of NBC1 and NBC2 with plasmid DNA. Figures 4(a)-(c) show that high levels of transfection can be obtained by increasing the proportion of the nucleic acid condensing peptide in the formulation. The experiment was performed as follows. Stock solutions of NBC1 and NBC2 were made up at concentrations of 2.5, 5 and 7.5 µg/120 µl of 25 mM Hepes. 0.85 M sodium chloride, pH 7.4 containing 0.7M sodium chloride. Each solution was mixed with 120 µl of 0.7 M sodium chloride 25 mM Hepes, pH 7.4 containing 2.5 µg RSVLUC plasmid DNA and the mixture incubated at room temperature for 30 mun followed by 4°C overnight. The synthetic virus like particles were then diluted into RPMI and incubated at room temperature for 30min before mixing with 1 x 10^6 Jurkat cells in RPMI containing 120 µM chloroquine. After 4h the cells were centrifuged, the medium removed and the cells resuspended in 2.5 ml of RPMI medium containing 10% fetal calf serum. After 24h the cells were collected, washed in 25 mM Hepes. 0.85 M sodium chloride, pH 7.4, lysed and the level of luciferase expression determined as described above.

Example 5

Comparison of Transfection Efficiency of Different NBC Peptides

In order to compare the relative efficiency of transfection of complexes composed of different NBC condensation peptides or poly-L-lysine, pRSVLuc DNA at a concentration of 100µg/ml was condensed using peptide at a ratio of 2µg/µg plasmid DNA as described in Example 4 with the following deviation: prior to addition to the cells [Jurkat cell line] the complexes were diluted to 20µg/ml with 10% PEG 10 000; 37mM sodium chloride; 25mM sodium phosphate, pH 7.4. The cells were assayed for luciferase after 24h.

These results are shown in Figure 5. Under these conditions all the peptides of low polydispersity including NBC14 (a synthetic poly-L-lysine of defined structure) were significantly better transfection agents than the polydisperse poly-L-lysine.

Figure 6a shows the results of a similar experiment. All conditions were identical to the previous example except that in this case the DNA was co-condensed with 0.6µgLip13 /µg DNA prior to dilution. Again significantly higher transfection rates were observed in all combinations apart from those containing poly-L-lysine. Figure 6b shows a similar comparison of data using NBC1, NBC8, NBC9 and NBC10 as condensing peptides.

Example 6

Gene Delivery Using Targeted Gene Complexes

6.1 Monoclonal antibody targeted gene transfer

6.1.1 Conjugate Synthesis

6.1.1.1 Conjugation of Monoclonal Antibody to NBC1 via the Carbohydrate Group

Cell targeting components such as monoclonal antibodies may be conjugated to a nucleic acid condensing peptide according to the invention.

The Carbohydrate groups present on the monoclonal antibody are oxidized using periodic acid to produce reactive aldehyde groups (Jentoft and Dearborn J. Biol. Chemi. 254. 4359 (1979)) . The oxidized antibody is then reacted with amino groups present on the NBC2 peptide. This results in the formation of an imine (Schiff Base) linkage between the antibody and the NBC polymer. The imine linkage is very labile in water and must be reduced using sodium cyanoborohydride or sodium borohydride to give the stable amine.

Anti-CD7 antibody (50 mg at 5 mg/ml) in 25 mM sodium acetate, pH 5.0 was oxidized by the addition of sodium periodate to 10 mM. The solution was left on ice, in the dark for 1h. The oxidized antibody was desalted and the buffer exchanged to 25 mM MES, pH 6.0 on a Sephadex G25 column (30 cm x 2.5 cm i.d.) . The amount of antibody recovered from the column was determined by measuring the absorbance at 280 nm (the absorbance of a 1 mg/ml solution of antibody at 280nm is 1.33). A 5 times molar excess of NBC1 was added and the solution was left at room temperature for 1h. Sodium cyanoborohydride was added to 10 mM and the solution was left at room temperature for a further four hours. before dialyzing overnight against 0.6 M sodium chloride; 25 mM HEPES, pH 7.9. in 12k molecular weight cut off membranes. The conjugate was removed from dialysis and loaded onto a SP Sepharose column (5 ml) equilibrated in 0.6 M sodium chloride; 25 mM HEPES pH 7.9. It was washed onto the column with 5 column volumes of the same buffer, then eluted with a gradient of 0.6 M to 3.0 M sodium chloride over 20 column volumes.

The unmodified antibody does not bind to the column and is washed through. The antibody conjugated to NBC1 binds to the column and is eluted early in the gradient (Fig. 7) . The conjugate peak eluted at approximately 1M NaCl.

6.1.1.2 Conjugation of Monoclonal Antibody to NBC2 via a Hydrazone and Disulfide Linkage

To 20 mg anti-CD33 antibody in 25 ml 25 mM sodium acetate, pH 5.0, sodium periodate was added to a final concentration of 10 mm. After 3 h at room temperature, the antibody was desalted by gel filtration using 25 mM sodium acetate, 0.5 M NaCl, pH 5.0, as running buffer. To the pooled protein fractions were added 5 mg (22 µmol) 3 - (2 -pyridyldithio) propionyl hydrazide (PDPH) dissolved in 50 µl DMSO. After 1 h at room temperature the antibody-PDPH was purified by Sephadex G-25 gel filtration using 25 mM HEPES, 0.5 M NaCl, pH 7.9, as running buffer. The protein fractions were pooled and stored at 4°C NBC-2-SH (5 mg/ml) was reduced with dithiothreitol (dithiothreitol) and purified by gel filtration using 25 mM HEPES, 0.5 M NaCl, pH 7.9, as running buffer. A total of 250 nmol NBC2 (as determined using an Ellman's test) were added to the antibody-PDPH and the solution was left for 16 h at room temperature. The concentration of NaCl was adjusted to 0.15 M before the antibody-NBC2 conjugate was purified by cation exchange on SP Sepharose using NaCl gradient of 0.15- 3 M. The separation obtained is shown in Fig. 4. The crude conjugate was applied to the column [S-Sepharose Fast Flow] on 0.15 M NaCl and eluted with a 0.15- 3 M linear gradient of NaCl. The conjugate peak eluted at approximately 1M NaCl. The protein peak was pooled and dialyzed against 0.02 M HEPES buffer, 0.15 M sodium chloride buffer, pH 7.2.

6.1.1.3 Conjugation of Monoclonal Antibodies to NBC2 via a Hydrazone and Thioether Linkage

To 20 mg anti-CD33 antibody in 2.5 ml of 25mM sodium acetate, pH 5.0, sodium periodate was added to a final concentration of 10 mM. After 3 h at room temperature in the dark, the antibody was desalted by gel filtration using 25 mM sodium acetate, 0.5 M NaCl, pH 5.0, as running buffer. To the pooled protein fractions were added 5 mg (22 µmol) 3-(2-pyridyldithio)propionyl hydrazide (PDPH) dissolved in 50 µl DMSO. After 1 h at room temperature the reduced antibody-PDPH was purified by Sephadex G-25 gel filtration using 25 mM HEPES, 0.5 M NaCl, pH 7.9, as running buffer. The protein fractions were pooled and stored at 4°C.

The derivatized antibody was reduced with cysteine and purified by gel filtration using 25 mM HEPES, 0.5 M NaCl, pH 7.9, as running buffer. The fractions were pooled and the concentration of thiol groups determined using an Ellman's test. A 2

molar excess of maleimido-β-Alanyl-NBC2- (S-acetamidomethyl-Cys) -COOH (5 mg/ml) were added to the antibody-PDPH and the solution was left for 16 h at room temperature. The concentration of NaCl was adjusted to 0.15 M before the antibody-NBC2 conjugate was purified by cation exchange on SP Sepharose using a NaCl gradient of 0.15 - 3 M. The protein peak was pooled and the pool sterile filtered and the conjugate stored at 4°C.

6.1.1.4 Synthesis of a Clustered Monoclonal Antibody-NBC2 Conjugate

Synthesis of Template Linker (2-bromoacetyl)-GG(E(NHNH$_2$)GG)$_4$-NHNH$_2$ (CL)

The peptide was synthesized using a Millipore 9050 plus peptide synthesizer equipped with enhanced counter-ion distribution monitoring to control the coupling time. Fmoc-Gly-O-Resin was used. After deprotection of the Fmoc group, using 20%, piperidine in dimethylformamide, the subsequent amino acids are coupled in four-fold excess using 0.6 M N,N'-diisopropylcarbodiimide with 0.025% Quinoline Yellow in dimethylformamide and 0.6 M 1-hydroxybenzotriazole with 1 mM diisopropylethylamine in dimethylformamide as activating agents. After the synthesis of the peptide was completed, the N-terminal Fmoc group was removed as described above and the peptide was cleaved from the resin using 95:5 TFA/water mixture. Purification of the peptide by gel filtration on Sephadex G25 followed by preparative reverse phase hplc gave the desired product.

The peptide may then be treated with 2-bromoacetic acid N-hydroxysuccinimidyl ester water to give the N-(2-bromoacetyl)-peptide. Followed by reaction of a solution of the peptide in water with a large excess of hydrazine in the presence of EDC-I to yield the N- (2-bromoacetyl) -penta-glutaryl-hydrazide derivative of the peptide.

Synthesis of a Clustered Monoclonal Antibody Ligand and its Conjugation to NBC2

To 20 mg anti-CD33 antibody in 2.5 ml 25 mM sodium acetate, pH 5.0, sodium periodate was added to a final concentration of 10 mM. After 3 h at room temperature in the dark, the antibody was desalted by gel filtration using 25 mM sodium acetate, 0.5 M NaCl, pH 5.0, as running buffer. To the pooled protein fractions were added 5 mg of peptide from (previous Example). After 1h at room temperature, the conjugate was purified by gel filtration and the peak pooled. A 2 molar excess of the thiol form of NBC2 was added to the clustered antibody derivative and the solution was

left for 16h at room temperature. The concentration of NaCl was adjusted to 0.15 M before the clustered antibody-NBC2 conjugate was purified by cation-exchange on SP Sepharose using a NaCl gradient of 0.15- 3 M. The protein peak was pooled and the pool sterile filtered and the conjugate stored at 4°C.

6.1.2 Targeted Gene Transfer Using Monoclonal Antibody Ligand Directed Gene Complexes

Examples of transfection efficiency are shown in Figs. 9-14. These figures are histograms showing the relative activity of luciferase in lysates derived from cells transfected with plasmid DNA containing the luciferase gene under the control of the RSV promoter. There is no endogenous luciferase activity in any of the cell lines or primary cells used.

Fig. 9 shows that gene transfer to myeloid cells can be targeted using an anti-CD33 complex in which the DNA is condensed with anti-CD33-NBC1 conjugate in a dose dependent manner. The anti-CD33-NBC1 conjugate was synthesized as described in Example 6.1.1.1. This experiment shows the effect of complex concentration on gene transfer. At 2.5µg/ml DNA complexed with unconjugated NBC2 no gene transfer was observed. As the amount of complex added to the cells is increased the level of luciferase (transfection) increases to a maximum of 1-2 µg/DNA complex/1 x 10^6 cells. Fig. 10 shows that the overall loading ratio of the plasmid with conjugate is important for activity with lower activity associated with complex prepared with an excess of conjugate. Anti-CD33-NBC1 complex was prepared such that the ratio of conjugate to DNA remained constant but the amount of unconjugated NBC2 was varied to give total cationic:phosphate ratios of 0.5 to 2.0.

Cell targeting is specific, as shown by the observation that cells treated with free and competing unconjugated anti-CD33 antibody have reduced levels of transfection when exposed to complexes formed using anti-CD33-NBC1 (prepared as described in Example 6.1.1.1) in the standard assay system (Fig. 11), and by the fact that no gene transfer is observed when cells are exposed to DNA condensed with unconjugated NBC2 (Fig 4(a)). In the competition experiment (Fig. 11), the standard assay described above was used except that the cells were incubated for 1h at 37°C with anti-CD33 antibody at the given concentrations just prior to addition of the synthetic virus like particle. The synthetic virus like particle was then added as normal and incubation continued for a further 3 h at 37°C. The presence of free antibody reduces the level of transfection presumably by preferentially interacting with th CD33 receptor.

Fig. 12 shows that the quantity of antibody within the complex can be optimized to increase transfection efficiency. Complexes were prepared as described in Example 4 with various proportions of anti-CD7-NBC1 conjugate (prepared as described in Example 6.1.1.1) and unconjugated NBC2. The final quantities were adjusted so that DNA condensation as determined by the gel retardation assay did not vary. Fig. 12 shows that when 25% of the condensation is provided by interaction between the nucleic acid and anti-CD7/peptide conjugate and 75% by free NBC2 maximal transfection efficiency is obtained.

Figure 13 shows the time course of expression. Cells were transfected with anti-CD7 conjugate prepared as described in Example 6.1.1.1 and assayed as described in Example 4 except that the cells were grown for increasing time periods before lysis and analysis of luciferase expression.

6.2 Insulin Targeted gene transfer

6.2.1 Synthesis of Insulin^{B1}-NBC14'

6.2.1.1 Functionalization of Insulin

Insulin was chemically modified according to previously described methods (pp.43-44 of Offord, R.E.(1980) 'Semisynthetic Proteins' 235pp., Wiley, Chichester and New York), with slight modifications. Briefly, 100mg Zn-free insulin were dissolved in 1ml of 1M sodium hydrogen carbonate, diluted with 4mL dimethylformamide and reacted with an equimolar amount (relative to peptide amino groups) of methylsulphonyloxycarbonic acid N-hydroxysuccinimide ester. After 1h incubation at room temperature, the mixture was acidified and subjected to semi-preparative HPLC on a C8 column equilibrated in 0.3M ammonium sulphate, pH 2.7, using a 65-90% gradient over 25min with a flow rate of 4mL/min (A:0.3M ammonium sulphate, pH 2.7. B: 0.3M ammonium sulphate containing 35% acetonitrile. The ammonium sulphate solutions were obtained by dilution of a 3M stock solution which had been adjusted to pH 2.7 (glass electrode) with conc. sulphuric acid. No further adjustment was made to the pH of the diluted solutions. The peak corresponding to di-substituted insulin (as judged by subsequent electrospray mass spectrometry) was collected and desalted on a double Chromabond equilibrated in 0.1% TFA. The di-derivative obtained in such reactions is known to be preponderantly the desired A1, B29 substituted molecule. This supposition was checked as follows: analysis of the

modified protein after overnight incubati n in 50mM dithi threitol in water allowed validation that the B-chain contained only a single methylsulphonyloxycarbonyl group (calcd., m/z 3547.8; found m/z, 3549.6 ± 0.4). 50mg methylsulphonyloxycarbonyl2-insulin were then dissolved in 1mL N-methyl-pyrrolidone and reacted with a 10-fold molar excess of Boc-AoA-OSu (synthesized according to Vilaseca et al. (1993) Bioconjugate Chem. 4 515-520), in the presence of equimolar amounts of HOBT and sufficient N-ethylmorpholine to bring the pH, as indicated using moist pH paper, to approximately 8. After 1h incubation at room temperature, the reaction medium was acidified and diluted with 0.1%TFA, and the derivatized insulin isolated by semi-preparative HPLC on a C8 column equilibrated in 0.1% TFA, using a 35-45% gradient (same eluants as described above) over 20min. The methylsulphonyloxycarbonyl groups were then cleaved under standard conditions and the material repurified on the C8 column using a 35-40% gradient over 20mins. The final compound, Boc-AoA-insulin, was characterized by electrospray mass spectrometry (calcd. m/z 5950.6; found m/z 5948.1 ± 0.1) and was deprotected by TFA treatment (30 minutes at room temperature) just before conjugation to NBC14.

6.2.1.2 Conjugation of Insulin^{B1}-NBC14^{1} Conjugate

The Cys-protected peptide was oxidized as follows. The peptide was dissolved in 50 mM imidazole (CI), pH 6.9 at a concentration of 10 mg/mL, and 0.2M methionine in water was added (as an anti-oxidant scavenger), a 10-fold molar excess over peptide. Then 50mM sodium periodate was added to a five-fold molar excess over peptide, and the solution allowed to stand in the dark for 5 minutes at room temperature, the mixture was purified by semi-preparative HPLC on a C8 column using a gradient of 10% to 60% solvent B over 25min where solvent A is 0.1% (w/v) aqueous TFA and Solvent B is TFA/ Acetonitrile-TFA-water 900:1:100 (V/W/V). The isolated oxidized peptide was dissolved into a solution of 5mg of the AoA-insulin derivative (an approx 2-fold molar excess of peptide over insulin) made up in 0.5mL 0.1M sodium acetate buffer to which had been added 50 µL acetonitrile, followed by adjustment to pH 3.8 (glass elctrode) with glacial acetic acid.

The conjugate was isolated after 15h incubation at room temperature and characterized by electrospray mass spectrometry (calculated, m/z 8426.1, found m/z 8429.3 ± 0.5). 4mg of material were isolated by semi-preparative HPLC with a 30-45 % gradient from the bulk of the reaction mixture. The peak fraction was dried down in a SpeedVac vacuum centrifuge. The final yield was 4mg of conjugate

6.2.2. Synthesis of a Conjugate Containing a Non-Receptor Binding Analogue of Insulin (des^{B23-31} Insulin^{B1}-NBC14^{1}

An insulin derivative which lacks the B23-31 sequence [des-insulin] was prepared as follows:

70mg of insulin were dissolved in 7.0ml of 50mM Hepes buffer, pH 8.0 and digested with 7mg of TPCK-trypsin for 3h at 37°C. The reaction was stopped by the addition of 140µl of a 0.1M solution of p-methyl sulphonyl fluoride in ethanol and the addition of acetic acid until a pH 3.0 was attained. The des-insulin was isolated by preparative rp-hplc using a Waters Nova-Pak HR C18 column and an aqueous trifluoroacetic acid (0.1%) to acetonitrile/trifluoroacetic acid/water (900:1:100) gradient (20-40% gradient over 40min). 38mg of material were recovered and characterized by electrospray mass spectrometry (calculated mass 4865.5 / observed mass 4867.7).

The introduction of an aminoxyacetyl group at the N-terminus of the B-chain of des-insulin was accomplished by an approach similar to that described by Offord (Offord, R.E.[1980]. Semi-synthetic Proteins. p 235. Wiley , London and New York). 7.7µM of des-insulin were dissolved in 0.5ml of 1M sodium hydrogen carbonate and diluted with 1.75ml of dimethyl formamide containing 9.4µM of methylsulphonyloxycarbonate N-hydroxysuccinimide ester (Tesser [1975] Peptides, pp 53-56. John Wiley). After 1 h incubation at room temperature the mixture was acidified with trifluoroacetic acid and the derivative re-chromatographed under the same conditions described above. The peak corresponding to the mono-substituted derivative of des-insulin (as judged by electrospray mass specrometry [calculated mass 5015, observed mass 5018]) was collected and freeze dried (25mg recovered). This material was dissolved in 0.4ml N-methyl-2-pyrollidone and reacted with a five fold molar excess of the N-hydroxysuccinimide ester derivative of Boc-aminooxyacetic acid [according to Vilasca et al [1993] Bioconjugate Chemistry 4. 515-520] in the presence of equimolar 1-Hydroxybenzotriazole (pH adjusted to 8.0 with N-ethylmorpholine). After incubation at room temperature for 1h the reaction was acidified and the insulin derivative purified as described above. The methylsulphonyloxycarbonyl group was then cleaved under standard conditions and the peptide repurified by hplc as described above. 8mg of aminooxyacetyl-des-insulin were recovered (calculated mass 4939 / mass observed by electrospray mass spectrometry 4938).

Periodate oxidized NBC14 was prepared as described in Example 6.2.1.2. 4mg of this material were mixed with 4mg of des-insulin derivative in 400µl of 0.1M sodium acetate buffer containing 20% acetonitrile, pH 3.6. After 15h incubation at room temperature the conjugate was isolated by preparative hplc using a 1cm diameter C8 column and a 30-35 % gradient of acetonitrile solvent (using Solvent A and Solvent B as described above). The recovered material (3.2mg) was characterized by electrospray mass spectrometry (calculated mass 7465 / observed mass 7462).

6.2.3 Preparation of Insulin-Poly-L-Lysine

This conjugate was prepared according to the protocol described by Birnstiel et al. (Meth Enzymol, 1993, 217, 618-644) for human transferrin-poly-L-lysine conjugate construction, with some slight modifications.

N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was reacted separately with both insulin and commercial poly-L-Lysine. The two derivatives were mixed after reduction of the 2-pyridyl disulphide group in modified poly-L-lysine.

20mg Zn-free insulin (3.5µmol) was solubilized in 200µL of 1M $NaHCO_3$, diluted with 800µL DMF and reacted with 2.2mg (7.0µmol) SPDP dissolved in 100µL DMF. After 1h incubation at room temperature, the mixture was acidified to pH 3.0 and subjected to semi-preparative HPLC on a C8 column equilibrated in 0.1% TFA using a 30-45%B gradient over 15min. with a flow rate of 4mL/min. The main derivative which was isolated (11mg) is the di-substituted bis (dithio-pyridine-propionyl)-insulin as judged by subsequent ESI-MS (calcd. m/z 6172.4, found m/z 6170.8$\pm$1.7).

20mg poly-L-lysine (MW 52.500, 0.38µmol) from Sigma dissolved in 1.9mL 0.1M HEPES buffer pH 7.9 was reacted with 3-molar excess of SPDP (1.14µmol dissolved in ethanol). After 30 min incubation the solution was acidified with 200µL 1M sodium acetate, pH4.6 and the modified polymer purified on a Pharmacia fast desalting column equilibrated in 20mM sodium acetate pH 5.0. The degree of substitution, was estimated at 4.3µmol dithiopyridine/µmol poly-lysine according to the amount of pyridine 2-thione released from an aliquot of material in the presence of 50mM DTT. PMSF (5mM, final c nc ntration) was added as a precaution against

protease contamination and the solution concentrated to 1.5L. 25mg DTT was added to modified poly-lysine and the pH adjusted to 7.0 with 1M HEPES buffer, pH7.9. After 30min, the solution was acidified with 1M sodium acetate, pH 4.6 and the material isolated on the fast desalting column under the same conditions described previously and concentrated.

0.18µmol of the thiol propionate (-SH) form of poly-L-lysine were finally recovered according to the quantitative ninhydrin test and mixed with a 5-molar excess of 3-(2-pyridyl dithio)- propionyl insulin and the pH adjusted to 7.0 with 1M HEPES, pH 7.9. After 1h incubation the conjugate was purified by gel filtration on a Superose 12 column equilibrated in 20mM sodium acetate, 0.1M NaCl, pH 5.0. The conjugate, corresponding to the first peak eluted from the column, was collected, dialysed against 1% acetic acid and water and finally freeze-dried (10mg recovered). This sample, corresponded to the material named Ins.PolyK1.

6.2.4 Targeted Gene Transfer using Insulin-NBC14 Conjugates

The insulin receptor targeted synthetic virus like particles were assembled using pRSVLuc plasmid DNA and Insulin-NBC14 conjugates as described in Example 4. Gene transfer efficiency was measured using the hepatic carcinoma cell line HepG2. These cells are adherent and the standard assay was modified as follows.
Cells were plated 24h prior to transfection in 6-well tissue culture plates at a density of 1-2 x 10^5 per well in complete medium (MEM (with Earle's salts) + 1% non-essential amino acids + 10% foetal calf serum) Transfection was carried out by aspirating the culture medium and washing the cells with phosphate buffered saline. Complex (50µg/ml DNA) is layered onto the cell monolayer in a 1ml final volume containing 2.5µg DNA and 120µM chloroquine in RPMI supplemented with human albumin (1mg/ml) and human transferrin (100µg/ml).The transfection is then allowed to proceed by incubating the cells at 37°C for 4h, after which the transfection complex was removed, the cells washed with PBS and completed medium added prior to incubation at 37°C for 24-72h in an atmosphere of 5% CO2.

The cells were then harvested using trypsin to detach the cells. After addition of medium containing serum to inactivate the trypsin, cells were pelleted by centrifugation at 13,000 rpm. The cells were then suspended in 1ml of PBS and re-centrifuged. The pellet was resuspended in 200µl of lysis buffer (100mM sodium phosphate, pH 7.8; 8mM MgCl2, 1mM EDTA; 1% Triton X-100;15% glycerol; 0.5mM PMSF and 1

mM Dithiothreitol) and agitated with a pipette. The lysate is centrifuged at 13,000 rpm for 1 minute and the supernatant collected. 80ml of the supernatant are transferred to a luminometer tube. The luciferase activity is then assayed using a Berthold Lumat L9501 luminometer. The assay buffer used is Lysis buffer containing 0.01mM Luciferin and 0.04375mM ATP. Light produced by the luciferase is integrated over 4sec and is described as relative light units (RLU). The data are converted to RLU / ml of lysate, RLU/cell or RLU/mg protein (protein concentration of the lysate having been determined in this case by the BioRAD Lowry assay).

Figure 14 shows the relative transfection potency of defined insulin-NBC14 conjugates and a defined conjugate of a non-receptor binding insulin analogue (des $^{B23-31}$ Insulin-NBC14) and insulin conjugates of poly-L-Lysine prepared by the approach of Birnstiel et.al. *loc cit*. These results show that targeting is specific and depends on the presence of an insulin conjugate which is able to recognize the receptor (InsI-NBC14 is active whilst des $^{B23-31}$ Insulin-NBC14 is totally inactive). Moreover significant transfection efficiency is dependent on precisely defined insulin-polycation conjugates as Insulin-NBC14 based gene delivery is at least 2 orders of magnitude higher than the best analogous insulin-poly-L-Lysine based delivery. The greatly reduced activity of the insulin-poly-L-lysine is due to largely to the poor transfection properties of poly-L-lysine. Insulin has 3 amino groups available for conjugation. In the defined NBC14 conjugates only one of these amino groups is used. Therefore, if conjugation via the other amino groups results in diminished receptor binding, it would be expected that the insulin-poly-L-Lysine conjugates would support up to 1/3 activity of the defined conjugates (the chemistry used to prepare the latter is random but group specific for amino groups). As the activity of these random conjugates is less than two orders of magnitude lower than the Insulin-NBC14 conjugates the additional loss in activity must be due to the inferior properties of polydisperse poly-L-Lysine polymers as transfection agents.

That delivery is targeted is shown in Figure 15 where addition of free insulin during the transfection process in the standard assay led to diminished gene transfection. It is inferred from this data that the presence of free unconjugated insulin can compete for the insulin receptor and thereby reduce the amount of insulin-targeted delivery particle taken up by the cells.

6.3 Glycosyl targeted gene transfer

6.3.1 Modification of NBC1 using 4(α-D-mannopyranosyloxy)phenylisothiocyanate (Man1-NBC1)

4(α-D-mannopyranosyloxy)phenylisothiocyanate (MPIC) was synthesized from *p*-aminophenyl-α-D-mannose according to the method of Muller and Schuber [*Biochem. Biophys. Acta*, 986, 97-105 (1989)].

10 mg (1.5μmol) NBC1 were dissolved in 1.8 ml 25 mM HEPES, pH 7.9. To this solution was added 10 mg (32 μmol) of MPIC dissolved in 50 μl DMSO. DMSO was then added dropwise to the stirred solution to dissolve any remaining insoluble MPIC. The mixture was left at room temperature for 1 h before purification by gel filtration using 25 mM Hepes, 1.5 M NaCl, pH 7.9, as running buffer. The eluted fractions were analysed quantitatively for carbohydrate using the phenol-sulphuric acid method [Robyt, JF and White, BJ (1987). *Biochemical Techniques: Theory and Practice*. Brooks/Cole, California.], and qualitatively for primary amine content using a ninhydrin test. The mannosylated NBC1 fractions were pooled and dialysed for 20 h against 25 mM HEPES, 0.15 M NaCl, pH 7.5. It was estimated that 30% of the lysyl residues of NBC1 were mannosylated using this method.

6.3.2. Use of Clustered Glycosyl Derivatives as Ligands for Targeting

Rationale for the design of glycosylated dendrimeric ligands.

Glycosyl moieties may be clustered by the use of branched carbohydrates of natural or synthetic origin. Alternatively these groups may be clustered by modifying a branched amino acid backbone (dendrimer).

The glycosylated dendrimeric moieties described in this section are based on a generic design of a ligand that, when conjugated to an NBC peptide, will give optimal binding of the peptide to either the hepatocyte asialoglycoprotein receptor (ASGPR) or the macrophage mannose receptor (MMR). The type of monosaccharide used in the synthesis of the ligand will depend on the receptor of interest. The design of the ligand is based on a consideration of the binding requirements of the ASGPR and the MMR, as it is these receptors that are of primary interest for use in gene targeting.

The literature states that ligands which present several (i.e three or more) monosaccharide units clustered together with their non-reducing ends exposed generally

provide optimal binding to membrane lectins, including the ASGPR and MMR (Lee and Lee (1987) Glycoconjugate J. 4, 317-328; Monsigny et al. (1994) Adv. Drug Del. Rev. 14, 1-24). Such ligands bind with more affinity than corresponding ligands presenting only one or two monosaccharide units - the so-called "cluster effect". Studies on the ASGPR have shown that the most successful ligands (in terms of binding affinity) are those which possess several monosaccharide moieties separated by an average distance of 1.5 nm. Theoretical calculations using "Hyperchem" modelling software shows that monosaccharide units such as those exemplified in the Man4Den2 (see below) structures are between 0.8 and 2.5 nm apart. In addition, Lee and Lee (1987) describe the optimal ligand for the ASGPR as having three galactose terminal residues with a triangular configuration, having intersite distances of 1.5, 2.2 and 2.5 nm. It is proposed that the flexibility and the inter-site distances of the monosaccharides present on the ligands exemplified are such as to allow our ligands to fit this criteria.

The binding requirements of the MMR have not been investigated as thoroughly as those for the ASGPR, although it is known that natural trisaccharides, which bind well to the MMR, present monosaccharides with intersite distances of between 1.0 and 3.0 nm. Again, it is proposed that the ligands exemplified fit such criteria.

6.3.2.1 Preparation of Mannosylated Lysine Dendrimer

The 2nd generation lysine dendrimer [Den2 (Lys)$_4$-Gly-Gly-Tyr-Cys] was synthesised using a Millipore 9050 Plus Pepsynthesiser running in extended cycle mode (1 h couplings) with TBTU / N-ethyldiisopropylamine activation and deprotection with 20 % Piperidine in dimethylformamide. Starting from Fmoc-Cys(Trt)-O-PEG-PS resin, and with a four-old excess of activated amino acid to free amine group, the peptide was synthesised sequentially using the following amino acid derivatives; Fmoc-O(*tert*-butyl)-tyrosine; Fmoc-glycine, di-Fmoc-lysine and Fmoc-N$^{\varepsilon}$-*tert*-butoxycarbonyl lysine. After the final coupling had finished the N-terminal Fmoc groups were removed using 20% piperidine in dimethylformamide.

The peptide was cleaved from the dried resin using reagent K (TFA, water, phenol,thioanisole,1,2-ethanedithiol; 82.5:5:5:5:2.5) and purified by ion-exchange (SP-sepharose)and reverse phase hplc.

To 15 mg (20 µmol) of the dendrimer in 1 ml of a 1:1 acetonitrile: water mixture, 6 mg (1.5 molar equivalents) of 2,2'-dipyridyldisulphide in 100 µl ethanol were added. After leaving the mixture for 5 min at room temperature, the peptide was purified by hplc.

10 mg (12 µmol) lyophilised peptide were dissolved in 1 ml of 1:1 acetonitrile: 0.1M borate, pH 9.0. The pH was adjusted to 9.0 by addition of NaOH. The peptide solution was then added to 95 mg 4-(α–D-mannopyranosyloxy)-phenylisothiocyanate dissolved in 1 ml of the acetonitrile/borate solution. The mannosylation was left to proceed at 37°C for 16 h.

The thiol of the mannosylated peptide was reduced by adding 20 mg solid dithiothreitol. The peptide was then purified by high resolution gel filtration.

By using appropriate spectrophotometric assays, the mannosylated peptide was determined to possess, on average, between 3 and 4 mannose groups per peptide molecule.

6.3.2.2 Conjugation of Mannosylated Dendrimer Ligand Man4Den2 to NBC12 (Synthesis of Conjugate Man_4DEN2-NBC12')

41.2 mg (10.2 µmol) NBC12 in 1.0 ml 50 mM imidazole, pH 6.9, were oxidised by the addition of 0.50 ml 0.1 M sodium periodate prepared in the imidazole buffer. After 5 min reaction at room temperature the reaction was stopped by the addition of 0.50 ml ethylene glycol (commercial solution). The medium was acidified pH 3.0 using acetic acid before purification by reverse phase HPLC. After lyophilization 30.0 mg oxidised NBC12 was obtained.

A maleimide function was introduced into NBC12 by addition of 4.3 mg (12.2 µmol) 4(-4-maleimidophenyl)butyric acid hydrazide. HCl. 1/2 dioxane to 10 mg (2.5 µmol) oxidised NBC12 in 2.0 ml 0.1 M sodium acetate, pH 4.6, containing 20% acetonitrile. After 15 h incubation at room temperature the hydrazone was purified by gel filtration in 1% acetic acid, pH 4.7. The peptide solution was lyophilised.

1.0 ml 50% acetonitrile/water containing 3.2 µmol of peptide (as determined by thiol analysis) was added to 5.0 mg (1.1 µmol) MAL-NBC12 in 0.1 M HEPES, pH 7.5, containing 20% acetonitrile. After 2 h incubation at room temperature the material was purified by reverse phase preparative HPLC and immediately lyophilised. The peptide construct was named Man_4Den2-NBC12'.

6.3.3 Preparation of Mannosylated Lysine Dendrimer - NBC12 Conjugate Man_4DEN5-NBC12'

Man_4Den5-NBC12 is a mannosylated dendrimer derivative of NBC-12, similar in structure of Man_4Den2-NBC12'. In this case however the mannosyl side chains are introduced using solid phase peptide synthesis coupling methods with the tetra-o-acetyl-mannose-α-Fmoc-Serine derivative.

The peptide dendrimers were synthesised using a Millipore 9050 Plus Pepsynthesizer as described above except that Fmoc-N^{ε}-*tert*-butoxycarbonyl lysine was replaced by di-Fmoc-lysine.

With the peptides on the resin, the four Fmoc-protected amine groups were deprotected using 20% piperidine in dimethylformamide. The free amines were then reacted with a tetra-O-Ac-mann-α-Fmoc-serine residue (in 16-fold molar excess of peptide) with TBTU/N-ethyldiisopropylamine as activating agent. After the coupling had finished, N-terminal Fmoc groups were removed using 2% DBU in dimethylformamide containing 2% piperidine.

The glycopeptides were cleaved from the resin by adding an excess of 95% TFA for 2 h at room temperature. The glycopeptide was precipitated in 10 volumes of ether, the ether discarded after centrifugation and purified by hplc.

In order to remove the O-acetyl protecting groups present on the carbohydrate moiety, the peptides were treated with NaOMe/MeOH before a final purification by HPLC.

The glycopeptide, Man4Den5 was coupled to NBC12 via a maleimide-hydrazide bifunctional crosslinker, as described above. Alternatively the glycopeptide was synthesised with a C-terminal hydrazide group by using a hydroxymethylbenzoic acid (HMBA) resin linker and cleavage method, as described in "synthesis of Man4Den4". Such hydrazide derivatives can be used to couple the Man peptide directly to oxidised NBC12, as described for the synthesis of Man4Den4-NBC12.

6) Biological Activity of Mannosylated NBC1.

In order to assess the potency of Man1- NBC1, transfer of the luciferase gene to HepG2 cells in culture was measured. The assay was carried out as described in Example 4 except the chloroquine concentration was increased from the standard 120μM to 240μM and the HepG2 cells were cultured for both 24h and 90h prior to enzyme assay.

The results of this assay are shown in Figure 16.

Example 7

Gene Transfer Complexes Containing Lipo-Peptide Complexes

7.1. N-Palmityl Derivatives

7.1.1 Synthesis of N-Palmityl NBC Derivatives

Palmitic acid N-hydroxysuccinimide ester (0.5g per gram of resin) was added to a suspension of N-terminal amino deprotected resin bound peptide (side chain protected)-O-PEG-PS resin in methanol/chloroform (1:4; 5ml per gram of resin synthesized as described in Example 2). The reaction was shaken for 48h. at room temperature then the resin was filtered off, washed 3 times with chloroform, washed with methanol and dried *in vacuo*.

The peptide was cleaved from the resin using a TFA/water/phenol/thioanisole/1,2-ethanedithiol (82.5: 5: 5: 5: 2.5) mixture (10ml of mixture for every gram of resin to be cleaved). The resin was then removed by filtration and washed 3 times with anhydrous trifluoroacetic acid. The combined filtrate and washings were concentrated by evaporation then precipitated using diethyl ether followed by centrifugation to give the crude peptide.

The crude peptide was dissolved in a small amount of 25mM HEPES pH 7.4 and applied to an SP-Sepharose fast flow column of an appropriate size and eluted using a gradient of 0-2M NaCl in 25mM HEPES pH 7.4 over 20 column volumes. The fractions containing the peptide, as shown by analytical reverse phase hplc, were pooled and applied directly to a preparative reverse phase hplc (Dynamax 83-221-C column) and eluted using an appropriate gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) (typically 30-50% acetonitrile over 20 min). The fractions containing the desired peptide were pooled, the acetonitrile evaporated *in vacuo* and lyophilised. The LIP-peptides were desalted by elution on a Sephadex G25 superfine column (1.6x30cm) with 1% acetic acid in water. The resulting fractions were analysed by reverse phase hplc and those containing pure peptide were pooled and lyophilised to give the final peptide.

7.1.2 Synthesis of Palmityl-Polylysine

Palmitic acid N-hydroxysuccinimide ester (25.7mg) was added to a stirred solution of Poly-(ε-Cbz)-lysine (molecular weight approximately 2000) (97.3mg) in dry dimethylformamide (1.5ml). The resulting solution was left to stir overnight at room temperature. Water (49ml) was added and the resulting precipitate spun down. The supernatant was decanted off and discarded and the pellet was frozen and lyophilised.

The remaining solid was dissolved in 30% HBr in acetic acid (6ml) and stirred for 30min at room temperature. Ether (44ml) was added and the resulting precipitate spun down. A second aliquot of ether (30ml) was added and the pellet resuspended and spun down again. The pellet was then taken up in water (15ml) and lyophilised.

7.1.3 Assembly of gene transfer complexes containing N-Palmityl Conjugates

Lipid containing complexes may be assembled as described in Example 4. The lipopeptide may be mixed with the NBC condensing peptide solution prior to the addition of the peptide mixture to the DNA solution. Alternatively the lipopeptide may be added to the complex after condensation with the NBC, before or after the dilution step prior to addition to the cells. In these cases, the complexes should be incubated overnight before addition of the lipopeptide.

7.1.4 Effect of N-Palmityl Peptides on Transfection Efficiency

7.1.4.1 Targeted Gene Delivery

Fig. 17 shows the effect of N-palmitoyl-NBC2 on transfection efficiency of a an anti-CD7-NBC2 conjugate. Complexes were made up as described in Example 4. The DNA was first treated with conjugate such that 50% of the material was condensed as determined using the gel retardation assay. Various mixtures of unconjugated NBC1 and N-Palmityl-NBC1 were addedsuch that complete condensation was obtained. These complexes were then employed in the standard assay system except that 100 μM chloroquine was used. As can be seen from Fig. 17, extremely high transfection efficiencies were obtained in. the presence of 12.5% N-Palmityl-NBC1. An increased efficiency of over 40-fold was observed for this conjugate.

Fig. 18 shows data similar to those shown in Fig 17 except the effect of N-palmitoyl-NBC1 on transfection efficiency of a an anti-CD33-NBC2 conjugate is shown.

7.1.4.2 Non-targeted Gene Delivery

The transfection efficiency seen when cells are treated with complexes assembled with a basic residue to phosphate ratio of greater than 1.0 (as described in Example 4 and shown in Fig 5) is increased significantly by the incorporation of palmitylated peptide in the complex.

Figure 19 shows that at an optimal ratio of Lip2 to NBC2 can boost transfection activity by > 2 orders of magnitude. Figure 20 shows that this effect is a common property of derivatized peptides of low polydispersity. These data also shows that peptides which have poor relative transfection efficiency (e.g. NBC8) when derivatized with an N-terminal palmityl group (e.g. Lip8) can form potent transfection agents in combination with other defined nucleic acid condensing peptides of low polydispersity. This, however, is not the case when these derivatized peptides are used in combination with poly-L-Lysine (Fig 21). In this case a derivatized peptide of low polydispersity (Lip13) failed to enhance transfection by a polymer of high polydispersity (poly-L-Lysine). This figure also shows that poly-L-lysine polymer derivatized with an N-terminal palmityl group as described above, enhances transfection by DNA condensed with peptide of low polydispersity at a significantly lower (<2 orders of magnitude) level than a derivatized peptide of low polydispersity (Lip13). This data also shows that a derivatized polymer of high polydispersity did not enhance transfection of DNA condensed with underivatized polymer.

The optimal ratios for transfection vary according to the target cell tissue. Fig 22 shows the differential transfection levels obtained with two different cell lines K562 and HepG2 and the same formulation Lip2 in NBC2. In the case of K562 cells little boost in activity was observed whereas in the case of HepG2 the optimal ratio was >1.0μg/μg DNA in the formulation. The optimum ratio for Jurkat cells is 0.13-0.6μg peptide/μg DNA.

The presence of palmitylated NBC peptides in the complex also confers higher resistance to inactivation by plasma. Figure 23 shows the effect on transfection efficiency of pre-incubating the synthetic virus like particle with varying amounts of

human plasma. The presence of Lip2 in the formulation clearly confers added resistance to this degradation. The NBC2 synthetic virus like particles were prepared essentially as described above using a DNA formulation of 1.5 µg NBC2 and were diluted into 25 mM Hepes, 0.85 M sodium chloride, pH 7.4 to a concentration of 10 µg DNA/ml. Lip2 synthetic virus like particles were prepared by mixing 1.5 µg Lip2/ µg DNA in 25 mM Hepes, 0.85 M sodium chloride, pH 7.4. The synthetic virus like particles were diluted into an equal volume of dilution/transfection medium containing 50%, 25%, 10% and 0% freshly prepared human plasma and 100 µM chloroquine. The solutions were then mixed with 1 x 10^6 Jurkat cells in RPMI containing 120 µM chloroquine. After 4h the cells were centrifuged, the medium removed and the cells resuspended in 2.5 ml of RPMI medium containing 10% foetal calf serum. After 24h the cells were collected, washed in 25 mM Hepes, 0.85 M sodium chloride, pH 7.4, lysed and the level of luciferase expression determined as described above.

7.2 Cholesteryl Derivatives

7.2.1 Synthesis of Cholesteryl NBC12 (Chol^{16}-NBC12^1)

7.2.1.1. Oxidation of NBC12

41.2 mg (10.2 µmol) NBC12 in 1.0 ml 50 mM imidazole, pH 6.9, were oxidised by the addition of 0.50 ml 0.1 M sodium periodate prepared in the imidazole buffer. After 5 min reaction at room temperature the reaction was stopped by the addition of 0.50 ml ethylene glycol (commercial solution). The medium was acidified pH 3.0 using acetic acid before purification by reverse phase HPLC. After lyophilisation 30.0 mg oxidised NBC12 were obtained.

7.2.1.2. Synthesis of cholesteryl 3' oxycarbohydrazide from cholesteryl chloroformate

To a vigorously stirred solution containing 200 mg (450 µmol) cholesteryl chloroformate in 8 ml 1:1 chloroform: methanol, 1.5 ml (20 mmol) hydrazine hydrate were added. After 1 h at room temperature a sample was taken for analysis by TLC, which indicated that the starting material (Rf = 0.45 in 8:1 hexane: chloroform solvent system) had been converted completely to a product (Rf = 0.00). 3 x 10 ml water was used to remove the hydrazine from the organic phase . Finally the organic solvent was

evaporated and the white solid obtained washed with ethanol. The product was dried for 2 h *in vacuo*. 110 mg of Cholesteryl 3'oxycarbohydrazide were obtained.

Correct synthesis was confirmed by electrospray mass spectrometry and IR analysis.

7.2.1.3. Synthesis of Chol^{3D}-NBC12'

40 mg (90 µmol) Cholesteryl 3'oxycarbohydrazide were dissolved in 0.8 ml chloroform and added to 0.8 ml MeOH containing 15.0 mg (3.8 µmol) oxidised NBC12. The reaction was left for 4 h at room temperature. The conjugate was isolated by preparative reverse phase HPLC using an acetonitrile/water solvent system containing 0.1% TFA. After evaporation of acetonitrile, the peptide conjugate was lyophilised. 6.7 mg of conjugate was obtained, which was stored at -80°C.

7.2.2 Biological Activity of Chol-NBC12'

The ability of cholesteryl NBC12 to condense DNA was checked as described in Example 4. The ability of this lipopeptide to promote gene transfer was measured by co-condensing RSVLuc plasmid DNA with NBC9 at the following peptide ratios:

DNA (µg)	NBC9 (µg)	LipoPeptide (µg)
1	2	-
1	2	0.15 Lip9
1	2	0.1 Chol^{3D}-NBC12'
1	2	0.4 Chol^{3D}-NBC12'
1	2	0.6 Chol^{3D}-NBC12'
1	2	1.0 Chol^{3D}-NBC12'

The formulations were prepared as follows:

2 µg NBC9 per µg of the final DNA amount required were mixed with the requisite amount of DNA such that the final DNA concentration was 100 µg/ml. The amount of $Chol^{\alpha}$-NBC12' to give the required concentration of lipidated peptide was then added to the complex and the complexes incubated for 1 h at room temperature. The buffer for all solutions was 0.6 M NaCl, 25mM phosphate buffer pH 7.4. After incubation overnight at 4°C the complexes were diluted to 20 µg/ml with a solution of 10% PEG 10000, 150µM chloroquine and 37mM sodium chloride and 25mM sodium phosphate buffer pH 7.4.

The complexes were assayed for the efficiency of luciferase gene transfer using Jurkat cells as described in Example 4. The results are shown in Fig 24.

7.3 Mannosyl-lipid-Conjugates

Combinations of lipid and targeting ligand may also be attached to the nucleic acid binding peptide.

7.3.1. Preparation of Mannosylated Dendrimer -NBC12 Conjugate with a Lipid Spacer (Man4Den3-NBC12')

This conjugate has a similar structure to Man_4Den2-NBC12' except the mannosylated lysine dendrimer ligand is separated from the nucleic acid binding peptide by an eleven carbon hydrocarbon spacer.

TMS-Cl(4.9ml, 38.7mmol) was added to a stirred suspension of aminoundecanoic acid(3.9g,19.37mmol) in dichloromethane(45.2ml) and the reaction was stirred for 15mins at RT. N-ethyldiisopropylamine (5.81ml) was added to the reaction and it was stirred for a further 15 min at RT then heated and refluxed for 1h. After cooling to RT 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl; 5.51g, 21.3mmol) was added to the

stirred solution and the reaction stirred for further 2h. The reaction mixture was then poured into water and shaken where upon the Fmoc-amino-undecanoic acid precipitated between the layers and could be filtered off and dried in vacuo to give (4.21g, 9.9mmol) of product.

The lysine dendrimer $Den_1(Lys)_4$-Gly-Gly-Tyr- aminoundecanoyl-Cys-was synthesised using a Millipore 9050 Plus Pepsynthesiser running in extended cycle mode (1 h couplings) with TBTU / N-ethyldiisopropylamine activation and deprotection with 20% piperidine in dimethylformamide. Starting from Fmoc-Cys(Trt)-O-PEG-PS resin, and with a four-old excess of activated amino acid to free amine group, the peptide was synthesised sequentially using the following amino acid derivatives; Fmoc-amino-undecanoic acid; Fmoc-O(*tert*-butyl)-tyrosine, Fmoc-glycine, di-Fmoc-lysine and Fmoc-N^{ε}-*tert*-butoxycarbonyl lysine. After the final coupling had finished the N-terminal Fmoc groups were removed using 20% Piperidine in dimethylformamide.

The peptide was cleaved from the dried resin using reagent K (TFA, water, phenol,thioanisole,1,2-ethanedithiol; 82.5:5:5:5:2.5) and purified by ion-exchange (SP-sepharose)and reverse phase hplc.

To 15 mg (20 µmol) of the dendrimer in 1 ml of a 1:1 acetonitrile: water mixture, 6 mg (1.5 molar equivalents) of 2,2'-dipyridyldisulphide in 100 µl ethanol were added. After leaving the mixture for 5 min at room temperature, the peptide was purified by HPLC.

10 mg (13 µmol) lyophilised peptide were dissolved in 1 ml of 1:1 acetonitrile: 0.1M borate, pH 9.0. The pH was adjusted to 9.0 by addition of NaOH. The peptide solution was then added to 95 mg 4-(α–D-mannopyranosyloxy)-phenylisothiocyanate dissolved in 1 ml of the acetonitrile/borate solution. The mannosylation was left to proceed at 37°C for 16 h.

The thiol of the mannosylated peptide was reduced by adding 20 mg solid dithiothreitol. The peptide was then purified by high resolution gel filtration.

By using appropriate spectrophotometric assays, the mannosylated peptide was determined to possess, on average, between 3 and 4 mannose groups per peptide molecule. The glycosylated lipid dendrimer was conjugated to NBC12 in an analagous way to that described in 6.3.2.1.

7.3.2 Insulin -NBC14^{1,20}-Cholesterol Conjugate

7.3.2.1Synthesis

A solution of thiocholesterol (0.5mg. 1.24µmol) in chloroform(100µl) was added to a stirred solution of the Insulin-NBC14^{1} (2mg. 0.24µmol) in methanol(400µl). The reaction was left to stir for 1h. at RT then 17h at 4°C. The reaction mixture was then evaporated to dryness, redisolved in water and the precipitate removed by centrifugation. The supernatant was applied to a PD-10 column (Pharmacia) and eluted with water. 0.75ml fractions were taken and fractions 3-7 combined and concentrated in a vacuum centrifuge to a volume of 1.5ml. The concentration of the final product was determined to be 0.39mg/ml(44.5µM) by its UV absorbance(0.23) at 275nm.

Example 8

Fusogenic Peptide-NBC Conjugates

8.1 Synthesis of Influenza HA Peptide Conjugates

A functional group is conjugated to a peptide of the invention as follows. One representative functional group is a fusogenic peptide. A fusogenic peptide (FPI3) derived from the Influenza HA may have the sequence:

NH_2-GLFEAIAGFIENGWEGMIDGGGC (Acm) - COOH

8.1.1 Synthesis of H_2N-FPI3- (S-acetamidomethyl-Cys) -COOH

The peptide was synthesized using a Millipore 9050 plus peptide synthesizer in extended synthesis cycle mode (1-hour couplings). Fmoc-Cys(Acm)-O-PEG-PS resin (Perseptive Biosystems Ltd.) was used. After deprotection of the Fmoc group, using 20% piperidine in dimethylformamide, the subsequent amino acids were coupled in four-fold excess using O-(lH-benzotriazol-i-yl)-tetramethyluronium tetrafluoroborate (TBTU)/l-hydroxybenzotriazole and N-ethyl-diisopropylamine as activating agents. After the synthesis of the peptide was finished, the N-terminal Fmoc group was removed as described above to give the free N-terminal amino side chain protected, peptide bound to the resin. This was cleaved from the resin using a TFA/water/phenol/-thioanisole/1,3-ethanedithiol (82.5:5:5:5:2.5) mixture. Following precipitation with ether and centrifugation the peptide can be purified using gel filtration to give the desired product.

The acetamidomethyl (Acm) thiol protecting group on the peptide may be removed using mercury (II) acetate with water/acetonitrile (1:1; 0.1% TFA) as solvent followed by precipitation of the mercury with 2-mercaptoethanol. The resulting free thiol peptide can be purified using gel filtration to give the desired product.

8.1.1.2 Synthesis of FPI3-NBC2[10]

NBC2 (thiol form) was synthesized by a standard solid state method and purified by hplc as described in Example 2. The peptide was treated with a 50 molar excess of 2,2'-dithiopyridyl disulphide and the S-pyridyl derivative purified by gel

filtration. A 5x molar excess of this intermediate was then reacted with the reduced thiol of the deprotected fusogenic peptide and the conjugate isolated by ion-exchange chromatography.

8.1.1.3 Synthesis of FPI3-NBC12'

The FP1 was derivatized with a two molar excess of bromoacetyl-hydrazide which places a hydrazide residue on the C-terminal thiol. After purification by hplc this FP1 intermediate was reacted with a 5 x molar excess of periodate oxidized NBC12. NBC12 was oxidized by the method described in Example 7.2.1.1. The resulting conjugate contains a hydrazone linkage between the FP and NBC2. Theoretically this linkage is acid-labile, and will be broken under the acidic conditions in the endosome, releasing free FP1 from the condensed DNA-conjugate complex. Alternatively the hydrazone group may be reduced with sodium borohydride to a stable hydrazide bond.

8.1.1.4 Synthesis of a fusogenic peptide containing a C-terminal protease-cleavable sequence FPI3.

The sequence of FP13 is :

NH_2-GLFEAIAGFIENGWEGMIDGGGF*LGEGGSC-COOH

This peptide was synthesized by standard solid phase automated methods as described in Example 8.1.1.

Demonstration of Endosome Protease Cleavage of FP13

The protease-cleavable sequence "GFLG" [Gly-Phe-Leu-Gly] present in the amino acid sequence of the peptide FPI3 (see above) was cleaved using the aspartic protease Cathepsin D. Cathespsin D activity is found in the endosomal compartment. Complexes containing conjugated FPI3 peptide may have increased transfection efficiency compared to those containing conjugated FPI3, since in theory the FPI3 sequence would be cleaved in the endosome, thus allowing th peptid to fuse with the

end somal membrane. The free peptide has been shown to have high fus genic activity [Wagner et al [1992] Proc.Natl.Acad.Sci U.S.A. 89, 7934-38.

The peptide was cleaved rapidly at pH 5.0 and very slowly at pH 7.0, as monitored by gel filtration analysis of the cleaved peptide.

The peptide bond (*) between the phenylalanine and leucine of the "GFLG" sequence is hydrolysed by Cathepsin D to give two fragments of molecular weight 2370 and 622. The larger N-terminal fragment has an absorbance at 280 nm but the smaller fragment does not. The cleavage reaction can therefore be monitored by measuring the A_{280} of the eluent obtained from high resolution gel filtration.

450 µl of 1 mg/ml peptide solution in 0.15 M NaCl was prepared using either 50mM sodium citrate, pH 5.0; 50mM sodium citrate, pH 6.0; or 50mM HEPES, pH 7.0. After preincubation at 37°C, 50 µl of Cathepsin D (EC 3.4.23.5) in water (1 mg/ml; 5-15 units/ml) were added and the solution mixed. [One unit will produce an increase in A_{280} of 1.0 per min per ml at pH 3.0 at 37°C measured as TCA-soluble products using haemoglobin as substrate (1 cm light path). After 30 and 90 min at 37°C 100 µl sub-samples were taken for immediate analysis by high resolution gel filtration using a Superdex Peptide HR 10/30 column (running buffer, HBS; flow rate, 0.75 ml/min; detection at 280 nm).

By analysing the A_{280} elution profile obtained from gel filtration each sample, it was found that the cleavage reaction was rapid at pH 5.0 and slower at pH 7.0.

pH	% peptide cleaved after:		
	0 min	30 min	90 min
5.0	0	80-85	95-100
6.0	0	30-35	--
7.0	0	1-5	--

This peptide may be conjugated t NBC peptides by the methods described in Example 8.1.., 8.1.2 and 8.1.3.

Example 9

Delivery of Enzymic Functionalities to Cells

9.1 Conjugation of Horseradish Peroxidase to NBC2

10 mg horseradish peroxidase was dissolved in 2 ml sodium acetate pH 5. Sodium periodate was added to 10 mM and the solution was left at room temperature fro 1 hour. The solution was desalted and the buffer exchanged to 25 mM MES pH 6 on a Pharmacia PD10 column. 2 mg NBC2 was added and the solution was left at room temperature for 1 hour. Sodium cyanoborohydride was added to 10 mM and the solution was left for 2 hours. At the end of this time glycine was added to 10 mM, the solution was left for a further 2 hours before dialysing overnight against 2 L 25 mM phosphate pH 7.4.

The conjugate was purified on a SP-sepharose column. The column was equilibrated in 25 mM phosphate pH 7.4 and the crude conjugate loaded. Unconjugated horseradish peroxidase washed straight through the column. The conjugate was eluted with a salt gradient of 0-1.5 M. The brown coloured fractions from the gradient were pooled and loaded onto a Sephacryl S-300 column equilibrated in 25 mM phosphate pH 7.4; 0.6 M NaCl. Once again the brown coloured fractions eluting from the column were pooled. The purified conjugate was too dilute for use straight from the column. Therefore it was dialysed against three changes of 5 L of distilled water and then freeze dried. The resulting powder was dissolved in approximately 0.8 ml water and filtered to remove insoluble material.

9.2 Transfection and Assay for Enzyme Delivery

The transfection assay described in Example 4 using Jurkat cells was employed with the following deviations from the generic method. 50µg DNA were condensed in 0.6M sodium chloride, 25mM sodium phosphate buffer, pH 7.4, 2% PEG 10 000 with 2.5mg of conjugate. After incubation overnight at 4°C the complex solution was diluted 1+4 with a solution of 37mM sodium chloride, 25mM sodium phosphate buffer, pH 7.4, 10% PEG 10 000 containing Lip13 at a concentration of 0.15µg/µg DNA. As usual 2.5µg DNA per assay point ($2x10^6$ cells) were used.

After 24h the cells were fixed in 0,5% glutraldhyde for 10 minutes at room temperature. The cells were then washed with phosphate buffered saline. The cells were then stained with Vectar Labs AEC (Vectar Labs Inc, Burlingaeme, CA. U.S.A.) for 10min. The stained cells were examined under an optical microscope x20 magnification. The damples treated with the conjugate showed a delivery frequency of approximately 20% as evidenced by complete black staining of the cells involved. No blacj staining was observed in the control sample.

Example 10

Gene Transfer using Nuclear tropic gene transfer complexes

Incorporation of peptide sequences which are known to control the import of proteins and nucleic acid-protein complexes can increase levels of transfection. These sequences can either be incoporated in the condensing peptide structure (NBC1 and NBC2) or in peptide domains which can be conjugated to condensing peptides.

10.1 Effect of Nuclear Localization Sequences Incorporated into Condensing Peptides.

NBC2 contains the SV40 T antigen nuclear localization signal. This sequence is known to promote the nuclear localization of proteins which contain the sequence (Roberts,B (1989) Biochim. Biophys. Acta 1008, 263). Peptide NBC5 has an identical structure to NBC2 except the sequence of the nuclear localization sequence is reversed. Reversal of the sequence motif is known to abolish nuclear localization properties.

The relative transfection potencies of NBC2 and NBC5 were compared in the standard non-targeting transfection assay (as described in Example 4) using Jurkat cells. Complexes were assembled in 0.6M sodium chloride with a ratio of 2µg NBC2 or NBC5 / µg pRSVLuc plasmid DNA. 0.15µg Lip13 were added to each complex before dilution in order to promote transfection

Results

Complex	Luciferase Activity	
	Duplicate 1	Duplicate 2
NBC2+Lip13	3,280,572	3,332,902

NBC5+Lip13	935,883	878,3

10.2 Synthesis of M9-NBC2

M9 includes the nuclear localization domain of hnRNP A1. The sequence of M9 is as follows:

M9 NH_2-TGNYNNQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGG YC-COOH

The sequence was modified by the addition of an N-terminal threonine residue and a C-terminal cysteine residue to permit chemical coupling to NBC2. The peptide was synthesized using a Millipore 9050 plus peptide synthesizer in extended synthesis cycle mode (1 hour couplings). Fmoc-Cys(Acm)-O-PEG-PS- was used. After deprotection of the Fmoc group, using 20% piperidine in dimethylformamide, the subsequent amino acids were coupled in four-fold excess using 0-(lH-benzotriazol-1-yl)-tetramethyluronium tetrafluoroborate (TBTU)/l-hydroxybenzotriazole and N-ethyl-diisopropylamine as activating agents. After the synthesis of the peptide was finished, the N-terminal Fmoc group was removed as described above to give the free N-terminal amino side chain protected peptide bound to the resin. This was cleaved from the resin using a TFA / water/phenol/ thioanisole/1,2-ethanedithiol (82.5:5:5:5:2.5) mixture. Following precipitation with ether and centrifugation, the peptide can be purified using gel filtration to give the desired product.

The acetamidomethyl (Acm) thiol protecting group on the peptide may be removed using mercury (II) acetate with water/acetonitrile (1:1; 0.1% TFA) as solvent followed by precipitation of the mercury with 2-mercaptoethanol. The resulting free thiol peptide can be purified using gel filtration to give the desired product.

Coupling of the M9 sequence to NBC2 is best achieved by oxidation of the M9 sequence with periodate to form an N-terminal aldehyde functionality. The thiol of NBC2 is deblocked and reacted with bromoacetylhydrazide. The hydrazide of NBC2 is then reacted with oxidized M9 and the conjugate purified by hplc.

10.3 Synthesis of Conjugates of HIV Matrix Protein and NBC12 (MAT1-NBC12)

HIV is a retrovirus which is able to infect non-dividing cells. The HIV Matrix protein is one of the proteins responsible for this property and the protein has two nuclear localization sequences and is thought to be responsible for transport of the virus through the nuclear pore (Gallay et al. (1995) Cell 80, 379; (1996) 83, 859). The sequence motifs thought to be responsible for the effect have been coupled in the design of a synthetic peptide sequence called MAT1 which has the following structure:

H-Thr-Gly-Lys-Lys-Lys-Tyr-Lys-Leu-Lys-His-Ile-Val-Lys-Ser-Lys-Lys-Lys-Ala-Gln-Gln-Ala-Ala-Ala-Asp-Thr-Gly-His-Ser-Ser-Gln-Val-Ser-Gln-Asn-Tyr-Cys(Acm)-COOH

The peptide was synthesised using a Biosearch 9050 plus Pepsynthesizer in extended synthesis cycle mode using Fmoc-Cys(Acm)-O-PEG-PS-Resin with DMF as solvent. Deprotection of the N-terminal Fmoc-group before each coupling was achieved using a solution of 20% piperidine in DMF(1min at a high flow rate followed by 10 min at 3ml/min). The amino acids were coupled in four fold excess using O-(1H-benzotriazo-1-yl)-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole and N-ethyldiisopropylamine as activating agents. The coupling times were set at 1hour throughout the synthesis. The following amino acid derivatives were used: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH Fmoc-Asp(O'Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH , Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser('Bu)-OH, Fmoc-Thr('Bu)-OH, Fmoc-Tyr('Bu)-OH, Fmoc-Val-OH. After the synthesis of the peptide was complete, the N-terminal Fmoc group was removed as described above to give the free amino, side chain protected, peptide bound to the resin. The resin was then washed into a glass vial with methanol and dried *in vacuo*.

The peptide was cleaved from the resin using a TFA/water/phenol/thioanisole/1,2-ethanedithiol (82.5: 5: 5: 5: 2.5) mixture (10ml of mixture for every gram of resin to be cleaved). The resin was then removed by filtration and washed 3 times with TFA.
The combined filtrate and washings were concentrated by evaporation then precipitated using diethyl ether followed by centrifugation to give the crude peptide.

The crude peptide was dissolved in a small amount of 1% acetic acid in water and applied to a Sephadex G25 (superfine) column of an appropriate size and eluted using the same 1% Acetic acid solution. The fractions containing the peptide, as shown by analytical reverse phase hplc, were pooled and lyophilised. Further purification was achieved by preparative reverse phase hplc using a Dynamax 83-221-C column and an appropriate gradient of Acetonitrile(0.1% TFA) in Water(0.1% TFA) over 20 min. The

fractions containing the peptide were pooled, the acetonitrile evaporated *in vacuo* and lyophilised.

The peptide was then desalted by elution on a sephadex G25 superfine column (1.6x30cm) with 1% acetic acid in water. The resulting fractions were analysed by reverse phase hplc, pooled and lyophilised as above.

The acetamidomethyl protecting group on the cysteine resin may be removed by treatment with Mercuric acetate in 30% acetic acid water. The product of this reaction may then be further purified by gel filtration on G25 as described above. The free thiol group may the be used as a handle to attach the peptide via a linker to an NBC. Alternatively oxidation of the N-terminal threonine moiety with periodate, as described for NBC12, furnishes the N-terminal glyoxal derivative which can be coupled to an appropriate peptide hydrazide.

10.4 Synthesis of conjugates of the antenapedia homeodomain and NBC12 (ANT1-NBC12)

The homeodomain of the Antennapedia gene product has been shown to have nuclear localization properties (Derossi et al [1994] J.biol.Chem. 269, 10444). The sequence of the domain thought to be responsible for this property has been identified and is incorporated in the following peptide:

H-Thr-Glu-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-Glu-Asn-Cys-COOH

The peptide was synthesised using a Biosearch 9050 plus Pepsynthesizer in extended synthesis cycle mode using Fmoc-Cys(Trt)-O-PEG-PS-Resin with DMF as solvent. Deprotection of the N-terminal Fmoc-group before each coupling was achieved using a solution of 20% piperidine in DMF(1min at a high flow rate followed by 10 min at 3ml/min). The amino acids were coupled in four fold excess using O-(1H-benzotriazo-1-yl)-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole and N-ethyldiisopropylamine as activating agents. The coupling times were set at 1hour throughout the synthesis. The following amino acid derivatives were used: Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(O'Bu)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH Fmoc-Phe-OH, Fmoc-Thr('Bu)-OH, Fmoc-Trp(Boc)-OH Fmoc-Val-OH. After the synthesis of the peptide was complete, the N-terminal Fmoc group was removed as described above to give the free amino, side chain

protected, peptide bound to the resin. The resin was then washed into a glass vial with methanol and dried in vacuo.

The peptide was cleaved from the resin using a TFA/water/phenol/thioanisole/1,2-ethanedithiol (82.5: 5: 5: 5: 2.5) mixture (10ml of mixture for every gram of resin to be cleaved). The resin was then removed by filtration and washed 3 times with TFA. The combined filtrate and washings were concentrated by evaporation then precipitated using diethyl ether followed by centrifugation to give the crude peptide.

The crude peptide was dissolved in a small amount of 25mM ammonium bicarbonate in water and applied to a Sephadex G25 (superfine) column of an appropriate size and eluted using the same 25mM ammonium bicarbonate in water. The fractions containing the peptide, as shown by analytical reverse phase hplc, were pooled and lyophilised.

The thiol group may be blocked/activated at this stage with Dipyridyldisulphide if desired.

Further purification was achieved by preparative reverse phase hplc using a Dynamax 83-221-C column and an appropriate gradient of Acetonitrile(0.1% TFA) in Water(0.1% TFA). The fractions containing the peptide were pooled, the acetonitrile evaporated *in vacuo* and lyophilised.

The peptide was desalted by elution on a sephadex G25 superfine column (1.6x30cm) with 25mM ammonium bicarbonate in water. The resulting fractions were analysed by reverse phase hplc, pooled and lyophilised as above.

Oxidation of the N-terminal threonine moiety with periodate, as described for NBC12, furnishes the N-terminal glyoxal derivative which can be coupled to an appropriate peptide hydrazide.

Example 11

Preparation of neutral hydophilic polymer coated synthetic virus like particles.

Neutral hydrophillic polymers such as PEG are known to effect the *in vivo* stability of particles and proteins in the blood stream. Incorporation of such polymers would therefore be of advantage in the development of parenteral gene delivery systems.

11.1 Synthesis of randomly derivatized PEG Nucleic acid condensing peptides

100 mg of lysine rich NBC peptide's or ε-amino blocked derivatives are dissolved in 100 ml of 25mM borate buffer, pH 8.8. The solid pegylation reagent, monomethoxy-polyethylene glycol-p- nitrophenyl carbonate, is weighed out to provide a 5-fold molar excess of reagent over available α- or ε - amino groups and dissolved in 100 ml water. The reagent solution is then added to the peptide solution and the mixture incubated for 3 hours at 37°C. At the end of this period, the reaction is quenched by addition of 10 ml of 100 mM ethanolamine. The pegylated peptide is purified by cation exchange chromatography as described in Example 2, and incorporated into the synthetic virus like particles as described in Example 4. The amount of pegylated peptide required to increase the *in vivo* transfection will vary with the cell type of the target cell.

11.2 Preparation of a Defined PEG Conjugate PEG_{5000}-NBC12[38]

A maleimide derivative of monomethoxy-PEG_{5000} (mPEG-MAL) was obtained from Sheatwater Polymers Inc., Huntsville, Al. U.S.A. and has been used to prepare a totally defined PEG-NBC9 conjugate. The single cysteine thiol of NBC12 was deprotected and reacted with the maleimide function on the PEG as follows: mPEG-MAL was added at a 5-fold molar excess to 15 mg of the thiol form of NBC12 in 25mM HEPES buffer, 0.15M sodium chloride (pH 7.4). The reaction was left at 25°C for 16h.

The following purification procedure was followed. A clear separation of free NBC12 and PEG_{5000} can be obtained using a gel permeation (Sephadex G-50) column. Gel permeation was therefore used to separate any unreacted NBC9 from the conjugate and unreacted mPEG-MAL. Cation exchange on SP-Sepharose was performed to separate unreacted mPEG-MAL from NBC12-S-MAL-PEG conjugate. A preliminary experiment confirmed that unreacted mPEG-MAL did not bind to the SP-Sepharose. The pure conjugate was desalted and lyophilised.

The conjugate is "heavier" by Superdex Peptide analysis than NBC12. That the conjugate is covalently linked is supported by evidence from gel filtration with the Superdex Peptide column using 6M Guanidine.HCl as running buffer.

11.3 Synthesis of a defined PEG-conjugate capable of targeting to glycosyl receptors

The presence of a PEG coating on the delivery complex (covalently linked) inhibits non-specific gene transfection (data not shown). For *in vivo* utility it is therefore important to incoporate any targeting ligand on the PEG polymer itself. In this way the ligand will be free of steric hinderance from the condensed DNA and shielding from the PEG group itself.

Conjugate which involve the mannose receptor ligand are described in Example 6.3.2. A ligand similar to Man_4Den5-NBC12, but with a PEG spacer group was synthesized as follows:

A two fold excess of Fmoc-NH-PEG(3400)-COOH is coupled to the HMB-PS resin using DCC and DMAP and recirculating overnight. The remaining amino acids (Fmoc-Gly-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Ser (O(Ac_4-mann))-OH and Fmoc-Tyr('Bu)-OH) are coupled in four-fold excess using the standard 1 hour coupling cycle with TBTU/N,N'-ethyldiisopropylamine activation and deprotection using 2% DBU in dimethylformamide containing 2% piperidine. After removal of the N-terminal Fmoc group on the completed resin bound peptide is treated with a solution of TFA/Water 95:5 for 1h. The peptide is then cleaved from the resin by stirring in a solution of 10% hydrazine in methanol for 1h. The glycopeptide was then be purified be reverse phase hplc, desalted and lyophilised. The sugar protecting acyl groups are removed as described in 6.3.2.2.

Example 12

Formulation of the Gene Delivery Complex

The formulation approach described in Example 4 and used for *in vitro* assay is not ideal for use as a pharmaceutical agent as this method (a) involves careful assembly of the gene delivery complex just before use and (b) limits the DNA concentration to 5µg/ml.

Two alternative types of formulation which do not require complicated preparation procedures and which are stable have been developed : these types of formulation are called High Salt Formulation and Isotonic Formulation. The Isotonic formulations require dilution with a solution, "the Diluent" just before administration. One of the major disadvantages of other methods of non-viral gene therapy, such as those involving the use of cationic liposomes or naked DNA is that the final product is not easily filter sterilized. This results in a requirement for aseptic processing during manufacture: an extremely expensive operation. The invention described here allows for easy filter sterilization by methods commonly used and validated in the pharmaceutical industry.

The High Salt Formulation is an *in vivo* formulation which involves direct injection of a hypertonic solution of the gene delivery vehicle. This formulation is therefore limited to direct injection of relatively small volumes <5ml into either specific tissues such as tumour tissue or i.v.

The Isotonic Formulation is useful for injection of large volumes or in *ex vivo* therapies.

12.1 Preparation of High Salt Formulations

These formulations contain 0.3-0.6mg/ml DNA and involve initial mixing of the requisite amount of condensing peptide or peptides (0.3-1.2mg/ml) with the DNA in the presence of 0.5-1M sodium chloride containing buffer [a physiologically acceptable buffer such as 25mM phosphate or HEPES], pH 6.5-8.0.
In the case of the HHS high salt formulations only the underivatized condensing peptide is added to the DNA. After incubation at 4°C for 16h other peptidic components and/or chloroquine are added as a concentrate in the same buffer.

In the case of the THS high salt formulation all peptidic components are added at the same time in the presence of buffer containing PEG (3000-10000). The PEG concentration is optimally 2% at a salt concentration of 0.6M and the DNA concentration 250µg/ml. These parameters were defined using the luciferase reported gene assay on Jurkat cells by varying the PEG and salt concentration (Figure 24) and at a fixed concentration of 2% PEG varying the DNA and salt concentration (Fig 25).

This formulation is particularly useful for the preparation of complexes which contain components which bind to filtration membranes. It has been shown that the presence of PEG 3000- 10000 prevents irreversible adsorption of Lip13 to the filtration membrane.

12.1.1 Preparation of a High Salt Formulation of pRSVLuc : Formulation HHS

The following solutions were prepared:

a) 800µg/ml of plasmid DNA in 0.7M sodium chloride, 25mM HEPES pH 7.4
b) 1.6 mg/ml NBC2 in 0.7M sodium chloride, 25mM HEPES, pH 7.4
c) 1mg/ml Lip2 in 0.7M sodium chloride, 25mM HEPES, pH 7.
d) 10mM Chloroquine in 0.7M sodium chloride, 25mM HEPES, pH 7.

The NBC2 solution (1 vol) was slowly added to the DNA solution (1vol) with agitation using a IKA-Schuttler MT4 machine [300rpm] at a rate of 0.1vol per minute. This operation was carried out at room temperature. After incubation for 1h at room temperature the complex was stored overnight at 4°C.

0.12ml of Lip2 solution per ml of complex was then added to the complex with agitation followed by 0.012ml of 10mM chloroquine solution per ml of complex solution. The complex was then incubated at 37°C for 30minutes and filter sterilized.

12.1.2. Preparation of a High Salt Formulation of pRSVLuc : Formulation THS

The following solutions were prepared:

a) 500µg/ml pRSVLuc DNA in 0.6M sodium chloride, 25mM sodium phosphate pH

7.4 and 2% PEG 10 000

b) 1mg/ml NBC13, 0.15mg Lip13 in 0.6M sodium chloride, 25mM sodium phosphate pH 7.4 and 2% PEG 10 000

c) 10mM chloroquine in 0.6M sodium chloride, 25mM sodium phosphate pH 7.4 and 2% PEG 10 000

The NBC13 and Lip13 solution (1 vol) was slowly added to the DNA solution (1vol) with agitation using a IKA-Schuttler MT4 machine [300rpm] at a rate of 0.1vol per minute. This operation was carried out at room temperature. After 1h at room temperature the compex was filter sterilized.

12.2. Isotonic Formulations

In these formulations the gene delivery complex is stored as a high salt formulation and diluted just prior to use.

It has been found that dilution into saline leads to physical instability and loss in transfection potency. It has been found that dilution into PEG not only leads to no loss in activity but leads to a significant, 3-10 fold boost in transfection potency (Figure 26a &b).

The effect of using PEG as an excipient has been shown to effect the size and size distribution of gene complexes

The Photon Correlation Spectroscopy (PCS) Analysis of NBC9 Synthetic virus like particles Prepared With and Without 3.25% PEG was as follows. NBC9 synthetic virus like particles containing 2µg peptide per µg DNA were prepared at a concentration of 100 µg DNA per ml in 0.8M sodium chloride with and without 3.25% polyethylene glycol (PEG) 8000 as described earlier. 200µg NBC9 in 1.0ml of 0.8 M NaCl 0.25mM HEPES pH 7.4 and in one case containing 3.25% PEG 8000 was shaken at 500rpm. 100 µg plasmid DNA was added in a small volume (54.6 µl). The complexes were left at room temperature for one hour then stored at 4°C overnight. PCS analysis was performed the following morning using a Malvern Instruments laser light scattering machine using S4700 v1.26 software and a cell adapted for a sample volume of 0.5ml. The laser angle was set at 90° and the average size calculated from 10 scans.

Table 1

Scan No.	Particle Diameter in nm	
	0.8M NaCl	0.8 M NaCl + 3.25% PEG
1	82.5	10.8
2	178	11.1
3	139.3	10.5
4	96.1	10.6
5	83.2	10.6
6	76.7	10.8
7	87.6	10.2
8	85.5	11.4
9	231	11.9
10	82.5	11.8
Average Size	114.2 +/- 52.2nm	11.0 +/- 0.6 nm

This experiment shows that in the presence of 3.35% PEG in the high salt formulation the gene delivery particles are significantly more compacted and regular shape.

12.2.1 Isotonic Formulation PEG1

The following solutions were prepared

a) 0.2mg/ml pRSVLuc DNA in 25mM sodium phosphate buffer, pH 7.4 containing 0.6M sodium chloride and 2% PEG 10 000.
b) 0.4mg/ml NBC13 and 0.12mg/ml Lip13 in 25mM sodium phosphate buffer, pH 7.4 containing 0.6M sodium chloride and 2% PEG 10 000.
c) 10% PEG 10000 in 25mM sodium phosphate buffer, pH 7.4 containing 37mM sodium chloride and 150µM chloroquine.

An equal volume of the DNA was added to the peptide solution at a rate of 0.1vol/ minute with mild agitation. This complex concentrate was then incubated at room temperature for 1h, filter sterilized and stored at 4°C until required for use.

Prior to assay or injection the concentrate was diluted with the PEG solution 1 volume of gene complex concentrate : 4 volumes of PEG diluent. The diluted preparation was then used within 24h.

12.2.2 Isotonic Formulation PEG2

a) 0.2mg/ml pRSVLuc DNA in 25mM HEPES buffer, pH 7.4 containing 0.6M sodium chloride and 2% PEG 10 000.

b) 0.4mg/ml NBC13 in 25mM HEPES buffer, pH 7.4 containing 0.6M sodium chloride and 2% PEG 10 000

c) 10% PEG 10000, 0.3µg/ml Lip13/ml, 1mg/ml human serum albumin, 50µg/ml human transferrin and 120µM chloroquine in RPMI 1640 culture medium.

An equal volume of the DNA was added to the peptide solution at a rate of 0.1vol/minute with mild agitation. This complex concentrate was then incubated at room temperature for 1h, filter sterilized and stored at 4°C until required for use.

Prior to assay or injection the concentrate was diluted with the PEG solution 1 volume of gene complex concentrate : 20 volumes of PEG diluent. The diluted preparation was then used within 24h.

Example 13

Shelf Stability of Formulated Synthetic Virus Like Gene Delivery Particles

A series of formulations of pRSVLuc DNA were prepared and stored in vials at -20°C and 4°C. At various time intervals the preparations were assayed for transfection efficiency using Jurkat cells as described in Example 4.

The formulations tested are as follows:

Stability Complexes

Complex	Formulation & Storage	Comments
S4	NBC13 2 µg/µg + 0.6 µg/µg LIP13 500 µg/ml 0.6 M NaCl 2% PEG 10000 +4 °C	Activity appears stable for greater than 40 days
S5	NBC13 2 µg/µg + 0.6 µg/µg LIP13 500 µg/ml 0.6 M NaCl 2% PEG 10000 -80 °C	
S6	NBC13 2 µg/µg 100 µg/ml 0.6 M NaCl +4 °C	S6-S9 are diluted 1/5 with 37.5 mM NaCl; 25 mM phosphate pH7.4; 10% PEG (10000 or 8000) containing LIP13 to 0.15 µg/µg immediately before use.
S7	NBC13 2 µg/µg 100 µg/ml 0.6 M NaCl -20 °C	
S8	NBC13 2 µg/µg 100 µg/ml 0.6 M NaCl 2% PEG 10000 +4 °C	
S9	NBC13 2 µg/µg 100 µg/ml 0.6 M NaCl 2% PEG 10000 -20 °C	
S10	NBC13 2 µg/µg + 0.15 µg/µg LIP13 100 µg/ml 0.6 M NaCl +4 °C	S10-S13 are diluted 1/5 with 37.5 mM NaCl; 25 mM
S11	NBC13 2 µg/µg + 0.15 µg/µg LIP13 100 µg/ml 0.6 M NaCl -20 °C	

S12	NBC13 2 µg/µg + 0.15 µg/µg LIP13 100 µg/ml 0.6 M NaCl 2% PEG 10000 +4 °C	phosphate pH 7.4; 10% PEG (10000 or 8000) immediately before use.
S13	NBC13 2 µg/µg + 0.15 µg/µg LIP13 100 µg/ml 0.6 M NaCl 2% PEG 10000 -20 °C	
S16	NBC13 2 µg/µg + 0.15 µg/µg LIP13 100 µg/ml 0.6 M NaCl 600 µM chloroquine +4 °C	S16-S19 are diluted 1/5 with 37.5 mM NaCl; 25 mM phosphate pH 7.4; 10% PEG (10000 or 8000) immediately before use.
S17	NBC13 2 µg/µg + 0.15 µg/µg LIP13 100 µg/ml 0.6 M NaCl 600 µM chloroquine -20 °C	
S18	NBC13 2 µg/µg + 0.15 µg/µg LIP13 100 µg/ml 0.6 M NaCl 600 µM chloroquine 2% PEG 10000 600 µM chloroquine +4 °C	
S19	NBC13 2 µg/µg + 0.15 µg/µg LIP13 100 µg/ml 0.6 M NaCl 2% PEG 10000 600 µM chloroquine -20 °C	

Figure 27 shows that in all except two cases the formulations are stable.

Example 14

Delivery of Oligonucleotides to Cells

The invention encompasses the delivery of oligonucleotides to cells in an analogous manner to plasmid DNA. Two 17 base oligonucleotides were synthesized:

Bcl 2S-B10

5'-GCGCACGCTGGGAGAAC-3'

Bcl AS-B10

5'-GTTCTCCCAGCGTGCGC-3'

Each oligonucleotide was synthesized on a phosphorothiolate backbone and was labeled with biotin in the 5' position. These oligonucleotides were obtained from R&D Systems Europe and relate to the 35-51 base coding region of the human BCl2 gene (Cleary et al., (1986); Cell, 47, 19-28); BCl-2S-B10 is a sense sequence oligonucleotide and BCl-AS-B10 is an antisense oligonucleotide. The latter oligonucleotide would be expected to interact with the natural human BC1-2 gene and by inhibiting BCl-2 gene expression induce apoptosis in tumorigenic cell lines such as Jurkat

Delivery of oligonucleotides was demonstrated by formulating BCl-2S-B10 and BCl-AS-B10 into a synthetic virus-like particle, as taught herein, and visualizing delivery to Jurkat cells

The oligonucleotides were condensed with 2µg NBCl3/1µg oligonucleotide (100µg oligonucleotides/ml) as described in Example 4 with the following modifications. The buffer was 25mM sodium phosphate pH 7.4, 0 6M with respect to sodium chloride. After 16 h at 4.0°C, the complex was diluted to 20µg oligonucleotide/ml in 10% PEG 10,000, 37.5mM sodium chloride, 25mM sodium phosphate buffer pH 6.5 containing 120µm chloroquine 0.15µg Lip13/µg oligonucleotide DNA 1 x 10^6 Jurkat cells per point were transfected with 2.5µg oligonucleotide DNA. The cells were incubated for 4 h as described in Example 4 prior to replacing the transfection medium with buffer. After 24 h the cells were washed with phosphate buffered saline and flushed for 10 min in 0.05% glutaraldehyde at room temperature. The cells were again washed with phosphate buffered saline and then permeabilized with 0.1% triton x 100 for 2 min at 4.0°C. After again washing with phosphate buffered saline the cells were treated with Vectorstain ABC according to the manufacturers instructions (Vector Laboratories, Burlington, C.A.). This kit contains an avidin (which bonds strongly to biotin), horseradish peroxidase conjugate. After 60 min at room temperature the cells were washed with phosphate buffered saline and stored with Vectorstain AEC. The

presence of horseradish peroxidase in the cells is indicated by a black color. After further washes with water the cells were examined at x 20 magnification by optical microscopy. The control cells were negative. Cells treated with synthetic virus-like particle containing oligonucleotides were positive (black) at a frequency of 20%.

Example 15

Transfection Efficiency *Ex Vivo* and *In vivo*.

15.1 *Ex-Vivo* Delivery

Particles of the invention are also capable of high level transfection of primary cells of the hematopoietic system (Figs. 28 and 29). In both cases the cells were incubated using the standard assay conditions except that 100 μM chloroquine was included in the transfection medium. Fig. 28 shows that the anti-CD33-NBC1complex can effect gene transfer to Peripheral Blood Mononuclear Cells at similar efficiencies to cell lines. The comparison shows the transfection efficiency of anti-CD33-NBC1 against the CD33+ cell line K562 and peripheral blood mononuclear cells prepared from peripheral blood by standard Ficoll gradient centrifugation Given the error between assays (% SEM-30%). the activities can be considered equivalent.

Fig. 29 shows that DNA complexed with anti-CD7-NBC1 can effectively transfer genes to fresh peripheral blood mononuclear cells and primary cultures of IL-2 stimulated T-Cells as well as cell lines (Jurkat) The comparison shows the activity of anti-CD7-NBC2 conjugates on the CD7+ cell line Jurkat (derived from a T-cell line) and fresh peripheral blood monocytes and peripheral blood monocytes activated by culturing under standard conditions in the presence of IL-2 for 7 days.

15.2 *In Vivo* Delivery

An important object of the invention is to transfer exogenous nucleic acid to mammalian cells *in vivo*. In this example, *in vivo* transfection of tumor cells by a Synthetic Virus like Particle of the invention is unequivocally demonstrated.

A murine carcinoma model was used to demonstrate the efficiency of the synthetic virus like particle *in vivo* Three BDF1 male mice (a strain developed by the Paterson Institute of Cancer Research. Manchester U.K. by crossing C57B16 with DBA2 mice) were implanted sub-cutaneously with cells of carcinoma line T50/80, a murine mammary carcinoma cell line which arose spontaneously in BDF1 mice (Paterson Institute; Dodd et al 1989 British J. Cancer 60, 164). 8 weeks after implantation each of the mice carried a 6-9mm diameter tumour mass on the right flank.

A solution of plasmid DNA (800μg/ml) coding for the β-galactosidase (lacZ) reporter gene in 25mM HEPES buffer containing 0.85mM sodium chloride was mixed at 300rpm using a vortex mixer (IKA-Schuttler MT4). An equal volume of 800 μg/ml

f peptide NBC2 in the same buffer was added dropwise to the DNA at a rate of 0.1 vol/min. The complex was incubated overnight at 4°C. Lip2 at a final concentration of 0.3µg/µg DNA was then added to the complex mixture and incubated at 37°C for 30 minutes.

Three mice bearing T50/80 tumors were anaesthetized with ether and the tumour mass injected with 20 µl of the following solutions: animal (a) HEPES buffer containing 7.14µg plasmid DNA; animal (b) delivery complex containing 7.14µg and animal (c) was injected with the same solution as animal (b) with an additional 0.24µl of a 10mM solution of chloroquine dissolved in the formulation buffer.

Mice were sacrificed by cerebral dislocation 48h after injection. Tumours were removed by dissection snap frozen in liquid nitrogen and sectioned (14µm sections cut through the center of the tumour mass) before fixing in 0.25% glutaraldehyde in phosphate buffered saline Sections were stained for β-galactosidase activity for 24h in X-GAL (Sigma Ltd., Poole U.K.) And counter stained with Nuclear Fast Red stain as described by Bout et al.(Exp. Lung Res 19, 193-202). In this assay cells expressing β-galactosidase activity stain blue

The slides were examined microscopically with the following results. Animal A (injected with naked DNA) all sections were essentially negative for β-galactosidase gene expression with only occasional blue staining of cellular debris in occasional sections: animal B (injected with complex): small patches of cells were stained in blue in occasional sections: animal C (injected with synthetic virus like particle): every section showed large areas of β-galactosidase positive cells confirming widespread dissemination of the complex throughout the tumour mass.

Representative sections of these tumours are shown in Fig. 24, which presents photographs taken of sections through tumour tissue transfected with plasmid DNA coding for the reporter gene lacZ, which leads to the expression of the enzyme β-galactosidase. The sections were prepared as described in the text and stained to show the location of cells expressing β-galactosidase. Slides were examined by and recorded using a standard microscope fitted with bright field optics. Slide 1 shows the results obtained from the tumour carried by Animal A which was injected with naked plasmid DNA. Slide 2 shows the results of the experiment performed with Animal B. This animal was injected with the complex prepared as described in the text. Slide 3 was taken from the tumour carried by Animal C whose tumour was injected with the same

complex as Animal B but was formulated in the presence of 120μM chloroquine. Arrows represent areas of staining.

Gene transfer to mammary carcinoma T50/80 using THS and PEG1 formulations

Four BDF1 male mice were implanted subcutaneously with cells of the T50/80 carcinoma line (Moore, Jpn. J. Cancer Res. 79, 236-243 (1988). 8 weeks after implantation each of the mice carried a tumour mass of diameter 6-9mm on the right flank.

To evaluate gene transfer of a sensitive reporter gene and a potential therapeutic gene to the same tumour tissue, two reporter plasmids were co-administered, one encoding the lacZ reporter and a second encoding the prodrug-converting enzyme nitroreductase, under the control of eukaryotic transcriptional regulatory elements.

A solution of plasmid DNA (pTK7.2, 200μg/ml) coding for the lacZ reporter gene in 25mM Hepes buffer, pH7.4, containing 0.85mM NaCl was vortexed at 300rpm using a vortex mixer (IKA-Schuttler MT4). An equal volume of peptide NBC13 at 400ug/ml in the same buffer was added dropwise to the DNA at a rate of 0.1 vol./min

A second solution of plasmid DNA (pBPV-ECO.NTR, 200ug/ml) coding for an expressible *E. coli* nitroreductase gene in 25mM Hepes buffer, pH7.4, containing 0.85mM NaCl was vortexed at 300rpm using a vortex mixer (IKA-Schuttler MT4). An equal volume of peptide NBC13 at 400ug/ml in the same buffer was added dropwise to the DNA at a rate of 0.1 vol./min

The complexes were incubated overnight at 4°C then equal volumes of each were mixed together. The 50:50 mixture was then diluted to 5μg DNA/ml into RPMI-based medium containing 50μg/ml human transferrin and 1mg/ml human serum albumin (RAT medium), with or without 10% PEG 8000. Optionally, LIP13 was then added to the complex mixture to a final concentration of 0.6μg LIP13 per μg DNA, and finally chloroquine was added to 120μM. These dual vector formulations were then administered to animals by injecting with 20μl complex, giving a total dose of 0.1μg plasmid DNA per tumour.

Tumour 1. DNA-NBC13 complexes diluted into RAT.
Tumour 2. DNA-NBC13 complexes diluted into RAT + 10% PEG 8000
Tumour 3. DNA-NBC13 complexes diluted into RAT + 10% PEG 8000 + 0.6 μg LIP13 / μg DNA

Tum ur 4. DNA-NBC13 complexes diluted into RAT + 0.6 µg/µg LIP13 / µg DNA

6 days after administration of complexes, animals were sacrificed by cerebral dislocation and tumours were dissected, snap frozen in liquid nitrogen and 14µm sections taken at 100µm intervals throughout the tumour with a cryostat microtome. Sections were mounted on microscope slides and air dried at room temperature. In order to estimate the translection efficiencies achieved with the above four formulations, sections were fixed in 0.25% glutaraldehyde/PBS, and then stained for β-galactosidase expression by incubating at 37°C in Xgal solution, as described by Bout et al. (Exp. Lung Res. 19, 193-202). In this assay cells expressing β-galactosidase activity stain blue. After staining overnight at 37°C in a humidified chamber, sections were dehydrated through an ethanol series, cleared in xylene and mounted in DPX mountant fluid.

Results

Fig. 31(a) shows that in Tumour 1, injection of DNA-NBC13 complexes diluted into RAT resulted in poor transfection efficiencies with only the occasional cell or small group of cells staining blue in just a few of all sections taken.

In Figure 31(b) it can be seen that injection of DNA-NBC13 complexes diluted into [RAT+10% PEG 8000] into Tumour 2 resulted in the transfection of only a few small clusters of cells or single cells in the tumour mass.

However, Figure 31(c) shows that injection of DNA-NBC13 complexes diluted into [RAT+10% PEG 8000 + 0.6μgLIP13/μgDNA] resulted in the efficient transfection of large groups of cells and these clusters were visible throughout the tumour mass, indicating that the complexes were well dispersed throughout the tumour upon injection. Many isolated single cells and clusters of cells were visible in sections taken at many different positions throughout the tumour, again indicating efficient dispersion of complexes upon injection and efficient gene transfer mediated by the delivery system.

Figure 31(d) shows that injection of DNA-NBC13 complexes diluted into [RAT+ 0.6μgLIP13/μgDNA] results in transfection of small groups of cells, and is less efficient than when this formulation is supplemented with 10% PEG 8000. Figure 31(e) shows a section of the same tumour in Figure 4 in which very efficient delivery to a layer of cells close to the surface of the tumour was observed with DNA-NBC13 complexes diluted into [RAT+0.6μgLIP13/μgDNA]. Only one such region was observed in this tumour, nevertheless the level of expression in this region was high.

Figure 31(f) shows a section of uninjected T50/80 tumour which stained negative for lacZ expression.

Conclusion

In vivo injection of gene delivery complexes, comprising both NBC13 peptides formulated in 10% PEG 8000, results in wide dispersion of complexes throughout the tumour mass, and efficient transfection in vivo of tumour cells.

Detailed description of figures

Figure 31(a):
Section of T50/80 mammary tumour tissue in which no lacZ$^+$ blue-staining cells can be seen.

Figure 31(b):
Section of T50/80 mammary tumour tissue in which a small cluster of lacZ$^+$ blue-staining cells can be seen in the centre of the field of view, occupying an area of ~1% of the total field of view.

Figure 31(c):
Section of T50/80 mammary tumour tissue in which a band of lacZ$^+$ blue-staining cells can be seen in the field of view extending from the top left-hand corner of the photograph towards the bottom right-hand corner. The blue cells are intermingled with unstained cells, but encompass an area of ~20% of the field of view. Approximately 30 foci of blue staining material, each focus representing a small cluster of cells or single cell can be seen in this figure. An area of faint blue staining can also be seen in the middle of the top edge of the figure. An area of faint blue staining can also be seen in the middle of the top edge of the figure, indicating a further patch of transfected cells.
(d) Section of T50/80 mammary tumour tissue in which an oval ring of lacZ$^+$ blue-staining cells can be seen in the field of view (ring diameter is ~40% of the horizontal dimension). The most strongly-staining lacZ$^+$ cells are at the bottom of the ring of cells, which is located in the centre of the field of view.

Figure 31(e):
Section of T50/80 mammary tumour tissue in which a small cluster of lacZ$^+$ blue-staining cells can be seen in the centre of the field of view, occupying an area of ~1% of the total field of view.

Figure 31(f):
Section of T50/80 mammary tumour tissue in which a layer of lacZ$^+$ blue-staining cells can be seen close to the surface of the tumour, occupying an area of ~10% of the field of view, and appearing as a dark vertical line to the right of the centre of the field of view.

Example 16
Dosage and Pharmaceutical Formulation

The nucleic acid condensing peptides and the nucleic acid to be delivered to cells may be formulated separately for parenteral administration or as the synthetic virus like particle. In the latter case the synthetic virus like particle may be assembled just prior to use. In the case of a pharmaceutical composition, the nucleic acid includes a gene whose expression would have some beneficial therapeutic effect on the cells of the recipient. For optimal efficiency of delivery of a therapeutic gene to a target cell, it is preferred that the therapeutic nucleic acid, in condensed form, be less than about 100nm, i.e., in the size range of approximately 1-100nm, or less than approximately 50kb in length. The nucleic acid may be in the form of plasmid DNA, either linear or circular, or in the form of a DNA fragment.

Examples of therapeutic genes are well known in the art and include but are not limited to the β-glucocerebrosidase gene, the Bruton's thymidine kinase gene, genes encoding cytokines, such as TNF, interleukins 1-12, interferons (α, β, γ), F_2 receptor, and T-cell receptor. The DNA may also include marker genes, such as drug resistance genes, the β-galactosidase gene, the dihydrofolate reductase gene, and the chloramphenicol acetyl transferase gene

The peptides and DNA are exchanged into isotonic phosphate free buffer and sterile filtered through a 0.45 or 0.22μ filter. The formulated solution or synthetic virus like particle (a mixture of the peptide conjugated to a selected functional group, DNA and free condensing peptide) may be sterile filled and aliquotted into suitable vials. The vials may be stored at 4°C, 20°C or 80°C or alternatively the DNA, peptide or synthetic virus like particle may be freeze dried from a buffer containing an appropriated carrier and bulking agent. In these cases, the dosage form is reconstituted with a sterile solution before administration

Use of this type of pharmaceutical composition in vivo or ex vivo with nucleic acid containing a gene of physiological importance, such as replacement of a defective gene or an additional potentially beneficial gene function, is expected to confer long term genetic modification of the cells and be effective in the treatment of disease.

For example, a patient that is subject to a viral or genetic disease may be treated in accordance with the invention via in vivo or ex vivo methods. For example in vivo treatments, a delivery vehicle of the invention can be administered to the patient, preferably in a biologically compatible solution or a pharmaceutically acceptable carrier, by ingestion, injection, inhalation or any number of other methods. The dosages

administered will vary from patient to patient; a "therapeutically effective dose" will be determined by the level of enhancement of function of the transferred genetic material balanced against any risk or deleterious side effects. Monitoring levels of gene introduction, gene expression and/or the presence or levels of the encoded anti-viral protein will assist in selecting and adjusting the dosages administered. Generally, a composition including a synthetic virus like particle will be administered in a single dose in the range of 10 ng - 100 ug/kg body weight, preferably in the range of 100 ng - 10 ug/kg body weight, such that at least one copy of the therapeutic gene is delivered to each target cell. The therapeutic gene will, of course, be associated with appropriate regulatory sequences for expression of the gene in the target cell.

Ex vivo treatment is also contemplated within the present invention. Cell populations can be removed from the patient or otherwise provided, transduced with a therapeutic gene in accordance with the invention, then reintroduced into the patient. In general, ex vivo cell dosages will be determined according to the desired therapeutic effect balanced against any deleterious side-effects. Such dosages will usually be in the range of 10^5-10^8 cells per patient, daily weekly, or intermittently; preferably 10^6- 10^7 cells per patient

A synthetic virus-like particle according to the invention may be used to treat X-linked γ-globulinemia The condensed nucleic acid in the virus-like particle will contain the Bruton's tyrosine kinase gene (Vetrie et al., 1993, Nature 361:226-233), which is carried on a 2.1 kb fragment delineated by the PvuI site at position (+33) and the HindIII site at position (+2126), if desired, the plasmid also may include sequences which confer position independent, tissue specific gene expression, as taught in PCT/GB88/00655. The therapeutic gene may also encode a splice site and poly A tail, which may include portions of the human β globin locus splice and poly A signals; i.e., a BamHI XbaI 2.8 kb 3' splice/poly A flanking sequence containing exon 2 IVSII - exon 3 - - polyA sequences

A synthetic virus-like particle containing the Bruton's tyrosine kinase gene is assembled as described herein and used to treat X-linked γ-globulinemia by introducing the construct directly into a patient for in vivo gene therapy or into pre-B cells for ex vivo therapy, as described in Martensson et al.; Eur. Jour. Immunol. 1987, 17:1499; Okabe et al., Eur. Jour. Immunol. 1992, 22:37; and Banerji et al., Cell 33:729, 1983, and administering the transfected pre-B cells into a patient afflicted with X-linked γ-globulinemia. A synthetic virus-like particle for treatment of X-linked γ-globulinemia will include a ligand for targeting of a preB cell. Such ligands are well-known in the art and will be specific for and capable of targeting one or more of

the following cell surface markers: CD9, CD10, CD19, CD20, CD22, CD24, CD38, CD40, CD72, and CD74.

A synthetic virus-like particle described herein also may be used for treatment of Gaucher's disease. Gaucher's disease stems from one of two different genetic mutations. Gaucher's type 1 is a CGG --> CAG mutation, which results in an Arg --> Gln substitution at position 119 of the :β-glucocerebrosidase polypeptide (Graves, DNA 7:521, 1988). Gaucher's type 2 is a CTG -> CCG mutation, which results in a Leu --> Pro substitution at position 444 of the Z-glucocerebrosidase polypeptide (Tsuji, NEJM 316:570, 1987). The presence of a :β-glucocerebrosidase gene encoding a wild type polypeptide is believed to substantially correct Gaucher's disease. Therefore, a therapeutic nucleic acid useful according to the invention includes the β-glucocerebrosidase gene, as described in Horowitz et al., 1989, Genomics 4:87-96, which is carried, as disclosed in Horowitz et al., on a 9722 base pair fragment extending from a BamHI site in exon 1 to an EcoRV site 31 to polyadenylation site. This fragment contains 11 exons and all intervening sequences, with translational start in exon 2. Sequences conferring position-independent and tissue-specific gene expression may be included in the construct and are carried on an 11.8 kb XhoI - SacI fragment from pIII.lyx construct as described in Bonifer et al., 1990, Euro. Mol. Biol. Org. Jour 9:2843.

A synthetic virus-like particle containing the β-glucocerebrosidase gene is assembled as described herein and used to treat Gaucher's disease by introducing the virus-like particle directly into the host for in vivo treatment, or into isolated macrophages for ex vivo therapy, as described in Immunology and Cell Biology, 1993, Vol. 71, pages 75-78 and introducing the transfected macrophages into a patient afflicted with Gaucher's disease. Expression of the wild type transgene in a patient afflicted with Gaucher's disease should result in correction of the diseased state. The synthetic virus-like particle will contain a ligand that specifically targets a cell surface antigen on a macrophage. Such ligands are well-known in the art, for example, monoclonal antibody having specificity for and capable of targeting one or more of the following cell surface markers: CD14, CD16, CD26, CD31, CDw32, CD36, CD45RO, CD45RB, CD63, CD71, CD74, CD23, CD25 and CD69.

The cells targeted for in vivo or ex vivo gene transfer in accordance with the invention include any cells to which the delivery of the therapeutic gene is desired. Such cells will bear a cell surface marker for which a corresponding specific ligand is available or can be prepared to allow for cell-specific targeting according to the invention. For example, cells of the immune system such as T-cells, B-cells, and macrophages, hemat poietic cells, and dendritic cells, each cell of which bears one or

more well-known cell surface receptors having corresponding ligands which may be selected for use as a targeting ligand in the virus- like particle of the invention, depending upon the selected cell. Using established technologies, stem cells may be used for gene transfer after enrichment procedures (see, for example, European Patent Applications 0 455 482 and 0 451 611, which disclose methods for separating stem cells from a population of hematopoietic cells). Alternatively, unseparated hematopoietic cells and stem cell populations may be used as a target population for DNA transfer as described herein.

Example 17

The following example describes transfection of mammalian cells using a synthetic virus like particle in which a lipidated nucleic acid condensing peptide is present, which particle was prepared according to a novel procedure which includes preincubation of the synthetic virus like particle with chloroquine.

In vitro transfection using the synthetic virus like particle is absolutely dependent on the presence of an agent which perturbs endosome function. One such potent agent is the anti-malarial drug chloroquine. The action of chloroquine can be amplified by pre-incubation of the synthetic virus like particles in a solution containing chloroquine prior to treatment of the cells (presumably by adsorption of this lipophilic molecule to the hydrophobic surface of the synthetic virus like particle). Figure 22 shows the increased transfection efficiency observed when synthetic virus like particles prepared in the presence of Lip2 are pre-incubated in the presence or absence of 120 μ M chloroquine before exposure to Jurkat cells. The synthetic virus like particle allows nucleic acid delivery to be optimized further by increasing binding of chloroquine through elevation of the level of lipophilic substituents in the synthetic virus like particle. The range of preincubation concentrations useful according to the invention are generally from 10 μM to 70mM. At the higher dosage, the maximum amount of chloroquine administered with the synthetic virus like particle in vivo should not exceed 3.5 mg/kg body weight. For ex vivo applications, the final concentration of chloroquine after dilution from the formulation should not exceed 200 μM.

Transfection efficiency in vitro can also be increased by extending the time period to which the target cells are exposed to the synthetic virus like particle in the presence of chloroquine. Figure 23 shows the effect of increasing the incubation time on transfection of Jurkat cells by Lip2 synthetic virus like particles in the presence of 120 μM chloroquine.

In Figure 23, solutions of NBC1 were either made up at a concentration of 200 μg/ml in 25 mM Hepes, 0.85 M sodium chloride, pH 7.4 containing 0.7 M sodium chloride or in the same buffer containing 5% PEG (Sigma Ltd., Poole, Dorset). Each solution was mixed with an appropriate amount of 3.5 mg/ml RSVLUC plasmid DNA to give a final concentration of 100 μg/ml plasmid DNA and the mixture incubated at room temperature for 60 min. followed by further incubation at 4°C overnight. The synthetic virus like particles were then diluted either directly into RPMI medium containing 1 x 10^6 Jurkat cells and 120 μM chloroquine (25 μl DNA

synthetic virus like particle into 1ml of cell culture medium) or into 25 mM Hepes, 0.85 M sodium chloride, pH 7.4 containing 10% PEG to a final concentration of 25 μg DNA/ml. After 30min at room temperature, the solutions in Hepes buffer were each diluted into RPMI containing 1 x 10^6 Jurkat cells and 120 μM chloroquine (100 μl DNA synthetic virus like particle into 1ml of cell culture medium). After 4h the cells were centrifuged, the medium removed and the cells resuspended in 2.5 ml of RPMI medium containing 10% fetal calf serum. After 24h, the cells were collected, washed, and lysed and the level of luciferase expression determined as described above.

OTHER EMBODIMENTS

Other embodiments will be evident to those of skill in the art. It should be understood that the foregoing detailed description is provided for clarity only and is merely exemplary. The spirit and scope of the present invention are not limited to the above examples, but are encompassed by the following claims.

CLAIMS

1. A synthetic virus like particle for transfecting nucleic acid into a mammalian cell, said synthetic virus like particle comprising

a recombinant nucleic acid,

a plurality of nucleic acid condensing peptides, said peptides being non-covalently associated with said recombinant nucleic acid such that said nucleic acid is in condensed form,

wherein each said nucleic acid condensing peptide is a heteropeptide, and said plurality of nucleic acid condensing peptides has low polydispersion.

2. The synthetic virus like particle of claim 1 wherein said plurality of nucleic acid condensing peptides comprise a first nucleic acid condensing peptide and a second nucleic acid condensing peptide, wherein said first nucleic acid condensing peptide comprises a first functional group covalently bound thereto.

3. The synthetic virus like particle of claim 2 wherein said second nucleic acid condensing peptide comprises a second functional group covalently bound thereto, said second functional group being different from said first functional group.

4. The synthetic virus like particle of claim 2, said first and said second nucleic acid condensing peptides having different amino acid sequences.

5. The synthetic virus like particle of claim 2, said first and said second nucleic acid condensing peptides having the same amino acid sequence.

6. The synthetic virus like particle of claim 2 wherein said first nucleic acid condensing peptide comprises a first functional group covalently bound thereto and further comprises a second functional group covalently bound thereto.

7. The synthetic virus like particle of claim 2 wherein said second functional group is covalently bound to said first functional group.

8. The synthetic virus like particle of claim 2 wherein said functional group comprises a ligand that is an antigenic peptide.

9. The synthetic virus like particle of claim 2 wherein said functional group comprises a ligand that targets a specific cell-type.

10. The synthetic virus like particle of claim 9, said ligand being selected from the group consisting of a monoclonal antibody, insulin, transferrin and asialoglycoprotein.

11. The synthetic virus like particle of claim 9 wherein said functional group comprises a ligand that targets cells in a non-specific manner.

12. The synthetic virus like particle of claim 11, said ligand comprising a sugar.

13. The synthetic virus like particle of claim 12 wherein said functional group comprises a lipid.

14. The synthetic virus like particle of claim 13, said lipid being selected from the group consisting of palmitoyl, oleoyl, stearoyl, and cholesterol.

15. The synthetic virus like particle of claim 2, said functional group comprising a neutral hydrophilic polymer.

16. The synthetic virus like particle of claim 15, said neutral hydrophilic polymer being selected from the group consisting of PEG and PVP.

17. The synthetic virus like particle of claim 2, said functional group being a fusogenic peptide.

18. The synthetic virus like particle of claim 17, said fusogenic peptide comprising HA peptide.

19. The synthetic virus like particle of claim 2, said functional group comprising an enzyme.

20. The synthetic virus like particle of claim 19, said enzyme being selected from the group consisting of a recombinase and an integrase.

21. The synthetic virus like particle of claim 2, said functional group comprising an intracellular trafficking protein.

22. The synthetic virus like particle of claim 21, said intracellular trafficking protein being selected from the group consisting of a nuclear localization sequence.

23. The synthetic virus like particle of claim 7 wherein said first functional group comprises one of a lipid or a neutral hydrophilic polymer and said second functional group is a ligand that targets a cell.

24. The synthetic virus like particle of claim23 wherein when said first functional group comprises a lipid, said second functional group comprises a ligand that targets a cellular receptor.

25 The synthetic virus like particle of claim 24, when said first functional group comprises PEG, said second functional group comprises a ligand that targets a cellular receptor.

26 . The synthetic virus like particle of claim 25, said ligand comprising one of a sugar moiety or a ligand whose cellular receptor is restricted to a cell-type.

27. The synthetic virus like particle of claim 23 , when said first functional group comprises a lipid, said second functional group comprises PEG.

28. The synthetic virus like particle of claim 1 wherein a said nucleic acid condensing peptide comprises an amino acid sequence of the generic formula

NH_2-A-$(X_1X_2Y_1Y_2)_nX_3X_4$-$(Z_1Z_2Z_3Z_4)$-$(X_5X_6Y_3Y_4)_mX_7X_8$BCOOH

wherein each of X_{1-8} is, independently, an amino acid having a positively charged group on the side chain;

wherein each of Y_{1-4} is, independently, a naturally occurring amino acid which promotes alpha helix formation;

wherein each of Z_{1-4} is, independently, a naturally occurring amino acid with at least 3 amino acids having a high propensity to form a stabilized turn structure;

wherein A is an amino-terminal serine or threonine residue;

wherein B is any amino acid; and

wherein n = 2 - 4 and m = 2.

29. The synthetic virus like particle of claim 28 wherein each of X_{1-8} is, independently, lysine, arginine, 2.4-diamino-butyric acid or ornithine.

30. The synthetic virus like particle of claim 28 wherein each of Y_{1-4} is, independently, glutamic acid, alanine, leucine, methionine, glutamine, tryptophan or histidine.

31. The synthetic virus like particle of claim 28 wherein each of Z_{1-4} is, independently, asparagine, glycine, proline, serine, and aspartic acid.

32. The synthetic virus like particle of claim 28 wherein B is any one of alanine, glutamic acid or cysteine.

33. The synthetic virus like particle of claim 28 wherein a said peptide comprises at least one of an internal serine, threonine, or cysteine residue.

34. The synthetic virus like particle of claim 33 a said internal residue is available for conjugation to a functional group.

35. The synthetic virus like particle of claim 28 wherein a said peptide comprises one of the amino acid sequences

(NBC7) H-TRRAWRRAKRRAARRCGVSARRAARRAWRRE-OH; and

(NBC11) H-TKKAWKKAEKKAAKKCGVSAKKAAKKAWKKA-$CONH_2$

36. The synthetic virus like particle of claim 1 wherein a said nucleic acid condensing peptide comprises a linear combination of the following three consensus sequences where the total sequence length is >17 residues:

Seq I:

-K-K-X-P-K-K-Y-Z-B-P-A-J-

wherein K is Lysine, P is Proline; A is Alanine; X is Serine, Threonine or Proline; Y is Alanine or Valine; Z is Alanine, Theonine or Proline; B is Lysine, Alanine, Threonine or Valine; and J is Alanine or Valine;

Seq II

-X-K-S-P-A-K-A-K-A-

wherein X is Alanine or Valine; K is Lysine; S is Serine; P is Proline; and A is Alanine.

Seq III

-X-Y-V-K-P-K-A-A-K-Z-K-B-

wherein X is Lysine or Arginine; Y is Alanine or Threonine; Z is Proline, Alanine or Serine; B is Lysine, Threonine or Valine; K is Lysine; P is Proline; A is Alanine.

37. The synthetic virus like particle of claim 36 wherein a said peptide comprises the amino acid sequence:

NH_2-[SV40 NLS]-[Seq I]-[Seq II]-[Seq III]-[SV40 NLS]-[Seq I]-C-COOH,

where -C- is Cysteine;where the SV40 NLS has the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Gln.

38. The synthetic virus like particle of claim 37 wherein a said peptide comprises the amino acid sequence

NH_2-PKKKRKVEKKSPKKAKKPAAKSPAKAKAKAVKPKAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-COOH.

39. The synthetic virus like particle of claim 36 wherein a said peptide comprises an amino acid sequence of the generic formula:

NH_2-X-$(Y)_n$-C-COOH

where X is absent or is one of serine or threonine;

Y is one of sequence I, II or III;

n is 2-6; and

C is cysteine.

40. The synthetic virus like particle of claim 39 wherein a said peptide comprises an amino acid sequence selected from the group of:

NH_2-[Seq III]-[SV40 NLS1]-[Seq I]-C-COOH,

where -C- is Cysteine;

NH_2-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine;

NH_2-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine; and

NH_2-[Seq I]-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine.

41. The synthetic virus like particle of claim 40 wherein a said peptide comprises an amino acid sequence selected from the group of sequences:

(NBC2) NH_2-KAVKPKAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-COOH;

(NBC8) NH_2-KKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-COOH;

(NBC13) NH_2-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-COOH; and

(NBC10) NH_2-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-COOH

42. A plurality of nucleic acid condensing peptides,

wherein each nucleic acid condensing peptide of said plurality being a heteropeptide, and said plurality of nucleic acid condensing peptides having low polydispersion,

said peptides being further characterized in that, when contacted with recombinant nucleic acid, the peptides are able to non-covalently associate with said nucleic acid to form a synthetic virus like particle containing condensed recombinant nucleic acid, and

said synthetic virus like particle being characterized in that, when contacted with a mammalian cell, said particle can transfect said nucleic acid into said cell.

43. The plurality of nucleic acid condensing peptides of claim 42, comprising a first nucleic acid condensing peptide and a second nucleic acid condensing peptide, wherein said first nucleic acid condensing peptide comprises a first functional group covalently bound thereto.

44. The plurality of nucleic acid condensing peptides of claim 43, wherein said second nucleic acid condensing peptide comprises a second functional group covalently bound thereto, said second functional group being different from said first functional group.

45. The plurality of nucleic acid condensing peptides of claim 38, said first and said second nucleic acid condensing peptides having different amino acid sequences.

46. The plurality of nucleic acid condensing peptides of claim 43, said first and said second nucleic acid condensing peptides having the same amino acid sequence.

47. The plurality of nucleic acid condensing peptides of claim 43 wherein said first nucleic acid condensing peptide comprises a first functional group covalently bound thereto and further comprises a second functional group covalently bound thereto.

48. The plurality of nucleic acid condensing peptides of claim 43 wherein said second functional group is covalently bound to said first functional group.

49. The plurality of nucleic acid condensing peptides of claim 43 wherein said functional group comprises a ligand that is an antigenic peptide.

50. The plurality of nucleic acid condensing peptides of claim 43 wherein said functional group

comprises a ligand that targets a specific cell-type.

51. The plurality of nucleic acid condensing peptides of claim 50, said ligand being selected from the group consisting of a monoclonal antibody, insulin, transferrin and asialoglycoprotein.

52. The plurality of nucleic acid condensing peptides of claim 50 wherein said functional group comprises a ligand that targets cells in a non-specific manner.

53. The plurality of nucleic acid condensing peptides of claim 52, said ligand comprising a sugar.

54. The plurality of nucleic acid condensing peptides of claim 53 wherein said functional group comprises a lipid.

55. The plurality of nucleic acid condensing peptides of claim 54, said lipid being selected from the group consisting of palmitoyl, oleoyl, stearoyl, and cholesterol.

56. The plurality of nucleic acid condensing peptides of claim 43, said functional group comprising a neutral hydrophilic polymer.

57. The plurality of nucleic acid condensing peptides of claim 56, said neutral hydrophilic polymer comprising PEG or PVP.

58. The plurality of nucleic acid condensing peptides of claim 43, said functional group being a fusogenic peptide.

59. The plurality of nucleic acid condensing peptides of claim 58, said fusogenic peptide comprising HA peptide.

60. The plurality of nucleic acid condensing peptides of claim 43, said functional group comprising an enzyme.

61. The plurality of nucleic acid condensing peptides of claim 60, said enzyme being selected from the group consisting of a recombinase and an integrase.

62. The plurality of nucleic acid condensing peptides of claim 43, said functional group comprising an intracellular trafficking protein.

63. The plurality of nucleic acid condensing peptides of claim 62, said intracellular trafficking protein being selected from the group consisting of a nuclear localization sequence.

64. The synthetic virus like particle of claim 48 wherein said first functional group comprises one of a lipid or PEG and said second functional group is a ligand that targets a cell.

65. The synthetic virus like particle of claim 64 wherein when said first functional group comprises a lipid, said second functional group comprises a ligand that targets a cellular receptor.

66 The synthetic virus like particle of claim 65, when said first functional group comprises PEG, said second functional group comprises a ligand that targets a cellular receptor.

67 The synthetic virus like particle of claim 66, said ligand comprising one of a sugar moiety or a ligand whose cellular receptor is restricted to a cell-type.

68. The synthetic virus like particle of claim 64 , when said first functional group comprises a lipid, said second functional group comprises PEG.

69. The plurality of nucleic acid condensing peptides of claim 42 wherein a said nucleic acid condensing peptide comprises an amino acid sequence of the generic formula

NH_2-A-$(X_1X_2Y_1Y_2)_nX_3X_4$-$(Z_1Z_2Z_3Z_4)$-$(X_5X_6Y_3Y_4)_mX_7X_8$BCOOH

wherein each of X_{1-8} is, independently, an amino acid having a positively charged group on the side chain;

wherein each of Y_{1-4} is, independently, a naturally occurring amino acid which promotes alpha helix formation;

wherein each of Z_{1-4} is, independently, a naturally occurring amino acid with at least 3 amino acids having a high propensity to form a stabilized turn structure;

wherein A is an amino-terminal serine or threonine residue;

wherein B is any amino acid; and

wherein n = 2 - 4 and m = 2.

70. The plurality of nucleic acid condensing peptides of claim 69 wherein each of X_{1-8} is, independently, lysine, arginine, 2.4-diamino-butyric acid or ornithine.

71. The plurality of nucleic acid condensing peptides of claim 69 wherein each of Y_{1-4} is, independently, glutamic acid, alanine, leucine, methionine, glutamine, tryptophan or histidine.

72. The plurality of nucleic acid condensing peptides of claim 69 wherein each of Z_{1-4} is, independently, asparagine, glycine, proline, serine, and aspartic acid.

73. The plurality of nucleic acid condensing peptides of claim 69 wherein B is any one of alanine, glutamic acid or cysteine.

74. The plurality of nucleic acid condensing peptides of claim 69 wherein a said peptide comprises at least one of an internal serine, threonine, or cysteine residue.

75. The plurality of nucleic acid condensing peptides of claim 74 a said internal residue is available for conjugation to a functional group.

76. The plurality of nucleic acid condensing peptides of claim 69 wherein a said peptide comprises one of the amino acid sequences

(NBC7) NH_2-TRRAWRRAKRRAARRCGVSARRAARRAWRRE-COOH; and

(NBC11) NH_2-TKKAWKKAEKKAAKKCGVSAKKAAKKAWKKA-$CONH_2$

77. The plurality of nucleic acid condensing peptides of claim 42 wherein a said nucleic acid condensing peptide comprises a linear combination of the following three consensus sequences

where the total sequence length is >17 residues:

Seq I:

-K-K-X-P-K-K-Y-Z-B-P-A-J-

wherein K is Lysine, P is Proline; A is Alanine; X is Serine, Threonine or Proline; Y is Alanine r Valine; Z is Alanine, Theonine or Proline; B is Lysine, Alanine, Threonine or Valine; and J is Alanine or Valine;

Seq II

-X-K-S-P-A-K-A-K-A-

wherein X is Alanine or Valine; K is Lysine; S is Serine; P is Proline; and A is Alanine.

Seq III

-X-Y-V-K-P-K-A-A-K-Z-K-B-

wherein X is Lysine or Arginine; Y is Alanine or Threonine; Z is Proline, Alanine or Serine; B is Lysine, Threonine or Valine; K is Lysine; P is Proline; A is Alanine.

78. The plurality of nucleic acid condensing peptides of claim 77 wherein a said peptide comprises the amino acid sequence:

NH_2-[SV40 NLS]-[Seq I]-[Seq II]-[Seq III]-[SV40 NLS]-[Seq I]-C-COOH,

where -C- is Cysteine;where the SV40 NLS has the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Gln.

79. The plurality of nucleic acid condensing peptides of claim 78 wherein a said peptide comprises the amino acid sequence

NH_2-PKKKRKVEKKSPKKAKKPAAKSPAKAKAKAVKPKAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-COOH.

80. The plurality of nucleic acid condensing peptides of claim 77 wherein a said peptide comprises an amino acid sequence of the generic formula:

NH_2-X-$(Y)_n$-C-COOH

where X is absent or is one of serine or threonine;

Y is one of sequence I, II or III;

n is 2-6; and

C is cysteine.

81. The plurality of nucleic acid condensing peptides of claim 80 wherein a said peptide comprises an amino acid sequence selected from the group of:

NH_2-[Seq III]-[SV40 NLS1]-[Seq I]-C-COOH,

where -C- is Cysteine;

NH_2-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine;

NH_2-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine; and

NH_2-[Seq I]-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine.

82. The plurality of nucleic acid condensing peptides of claim 81 wherein a said peptide comprises an amino acid sequence selected from the group of sequences:

(NBC2) NH_2-KAVKPKAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-COOH;

(NBC8) NH_2-KKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-COOH;

(NBC9) NH_2-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-COOH; and

(NBC10) NH_2-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKP AAC(Acm)-COOH

83. A synthetic virus like particle for transfecting nucleic acid into a mammalian cell, said synthetic virus like particle comprising

a recombinant nucleic acid,

a first plurality of first nucleic acid condensing peptides, each said peptide comprising a covalently linked first functional group,

a second plurality of second nucleic acid condensing peptides,

wherein each said nucleic acid condensing peptide is a heteropeptide and each said plurality of nucleic acid condensing peptides has low polydispersion,

wherein each said plurality of nucleic acid condensing peptides is non-covalently

associated with said recombinant nucleic acid such that said nucleic acid is in condensed form.

84. The synthetic virus like particle of claim 83, further comprising a third plurality of third nucleic acid condensing peptides, each said third peptide comprising a covalently linked second functional group which is different from said first functional group.

85. The synthetic virus like particle of claim84, said first and second functional groups are present in said synthetic virus like particle in a preselected ratio.

86. The synthetic virus like particle of claim 83, said first and said second nucleic acid condensing peptides having different amino acid sequences

87. The synthetic virus like particle of claim 83, said first and said second nucleic acid condensing peptides having the same amino acid sequence.

88. The synthetic virus like particle of claim 83 wherein said first nucleic acid condensing peptide comprises a first functional group covalently bound thereto and further comprises a second functional group covalently bound thereto.

89. The synthetic virus like particle of claim 88 wherein said second functional group is covalently bound to said first functional group

90. The synthetic virus like particle of claim 83 wherein said functional group comprises a ligand that is an antigenic peptide.

91. The synthetic virus like particle of claim 83 wherein said functional group comprises a ligand that targets a specific cell-type.

92. The synthetic virus like particle of claim 91, said ligand being selected from the group consisting of a monoclonal antibody, insulin, transferrin and asialoglycoprotein.

93. The synthetic virus like particle of claim 91 wherein said functional group comprises a ligand that targets cells in a non-specific manner.

94. The synthetic virus like particle of claim 93, said ligand comprising a sugar.

95. The synthetic virus like particle of claim 94 wherein said functional group comprises a lipid.

96. The synthetic virus like particle of claim 95, said lipid being selected from the group consisting of palmitoyl, oleoyl, stearoyl, and cholesterol.

97. The synthetic virus like particle of claim 93, said functional group comprising a neutral hydrophilic polymer.

98. The synthetic virus like particle of claim 97, said neutral hydrophilic polymer comprising PEG or PVP.

99. The synthetic virus like particle of claim 83, said functional group being a fusogenic peptide.

100. The synthetic virus like particle of claim 99, said fusogenic peptide comprising HA peptide.

101. The synthetic virus like particle of claim 83, said functional group comprising an enzyme.

102. The synthetic virus like particle of claim 101, said enzyme being selected from the group consisting of a recombinase and an integrase.

103. The synthetic virus like particle of claim 83, said functional group comprising an intracellular trafficking protein.

104. The synthetic virus like particle of claim 103, said intracellular trafficking protein being selected from the group consisting of a nuclear localization sequence.

105. The synthetic virus like particle of claim 89 wherein said first functional group comprises one of a lipid or PEG and said second functional group is a ligand that targets a cell.

106. The synthetic virus like particle of claim 105 wherein when said first functional group comprises a lipid, said second functional group comprises a ligand that targets a cellular receptor.

107. The synthetic virus like particle of claim 106, when said first functional group comprises PEG, said second functional group comprises a ligand that targets a cellular receptor.

108. The synthetic virus like particle of claim 107, said ligand comprising one of a sugar moiety or a ligand whose cellular receptor is restricted to a cell-type.

109. The synthetic virus like particle of claim 105 , when said first functional group comprises a lipid, said second functional group comprises PEG.

110. The synthetic virus like particle of claim 83 wherein a said nucleic acid condensing peptide comprises an amino acid sequence of the generic formula

NH_2-A-$(X_1X_2Y_1Y_2)_nX_3X_4$-$(Z_1Z_2Z_3Z_4)$-$(X_5X_6Y_3Y_4)_mX_7X_8$BCOOH

wherein each of X_{1-8} is, independently, an amino acid having a positively charged group on the side chain;

wherein each of Y_{1-4} is, independently, a naturally occurring amino acid which promotes alpha helix formation;

wherein each of Z_{1-4} is, independently, a naturally occurring amino acid with at least 3 amino acids having a high propensity to form a stabilized turn structure;

wherein A is an amino-terminal serine or threonine residue;

wherein B is any amino acid; and

wherein n = 2 - 4 and m = 2.

111. The synthetic virus like particle of claim 110 wherein each f X_{1-4} is, independently, lysine, arginine, 2.4-diamino-butyric acid or ornithine.

112. The synthetic virus like particle of claim 110 wherein each of Y_{1-4} is, independently, glutamic acid, alanine, leucine, methionine, glutamine, tryptophan or histidine.

113. The synthetic virus like particle of claim 110 wherein each of Z_{1-4} is, independently, asparagine, glycine, proline, serine, and aspartic acid.

114. The synthetic virus like particle of claim 110 wherein B is any one of alanine, glutamic acid or cysteine.

115. The synthetic virus like particle of claim 110 wherein a said peptide comprises at least one of an internal serine, threonine, or cysteine residue.

116. The synthetic virus like particle of claim 115 wherein a said internal residue is available for conjugation to a functional group.

117. The synthetic virus like particle of claim 110 wherein a said peptide comprises one of the amino acid sequences

(NBC7) H-TRRAWRRAKRRAARRCGVSARRAARRAWRRE-OH; and

(NBC11) H-TKKAWKKAEKKAAKKCGVSAKKAAKKAWKKA-NH_2

118. The synthetic virus like particle of claim 83 wherein a said nucleic acid condensing peptide comprises a linear combination of the following three consensus sequences where the total sequence length is >17 residues:

Seq I:

-K-K-X-P-K-K-Y-Z-B-P-A-J-

wherein K is Lysine, P is Proline; A is Alanine; X is Serine, Threonine or Proline; Y is Alanine or Valine; Z is Alanine, Theonine or Proline; B is Lysine, Alanine, Threonine or Valine; and J is Alanine or Valine;

Seq II

-X-K-S-P-A-K-A-K-A-

wherein X is Alanine or Valine; K is Lysine; S is Serine; P is Proline; and A is Alanine.

Seq III

-X-Y-V-K-P-K-A-A-K-Z-K-B-

wherein X is Lysine or Arginine; Y is Alanine or Threonine; Z is Proline, Alanine or Serine; B is Lysine, Threonine or Valine; K is Lysine; P is Proline; A is Alanine.

119. The synthetic virus like particle of claim 118 wherein a said peptide comprises the amino acid sequence:

NH_2-[SV40 NLS]-[Seq I]-[Seq II]-[Seq III]-[SV40 NLS]-[Seq I]-C-COOH,

where -C- is Cysteine;where the SV40 NLS has the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Gln.

120. The synthetic virus like particle of claim 119 wherein a said peptide comprises the amino acid sequence

H-PKKKRKVEKKSPKKAKKPAAKSPAKAKAKAVKPKAAKPKKPKKKRKVEKKSP KKAKKPAAC(Acm)-OH.

121. The synthetic virus like particle of claim 118 wherein a said peptide comprises an amino acid sequence of the generic formula:

NH_2-X-$(Y)_n$-C-COOH

where X is absent or is one of serine or threonine;

Y is one of sequence I, II or III;

n is 2-6; and

C is cysteine.

122. The synthetic virus like particle of claim 121 wherein a said peptide comprises an amin acid sequence selected from the group of:

NH_2-[Seq III]-[SV40 NLS1]-[Seq I]-C-COOH,

where -C- is Cysteine;

NH_2-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine;

NH_2-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine; and

NH_2-[Seq I]-[Seq I]-[Seq I]-[Seq I]-C-COOH

where -C- is Cysteine.

123. The synthetic virus like particle of claim 122 wherein a said peptide comprises an amino acid sequence selected from the group of sequences:

(NBC2) H-KAVKPKAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-OH;

(NBC8) H-KKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-OH;

(NBC9) H-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-OH; and

(NBC10) H-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKP AAC(Acm)-OH

124. A method of transfecting a mammalian cell with a recombinant nucleic acid, said method comprising contacting a mammalian cell with a synthetic virus like particle as claimed in claim 1.

125. A method of transfecting a mammalian cell with a recombinant nucleic acid, said method comprising the steps of

a) forming a mixture of a synthetic virus like particle as claimed in claim 1 and an endosomal disruption agent, and

b) contacting said mixture of step a) with a mammalian cell under conditions sufficient to allow transfection of said cell with said nucleic acid.

126. A method of formulating a synthetic virus like particle for administration to a patient, the method comprising mixing a synthetic virus like particle as claimed in claim 1 with an endosomal disruption agent.

127. The method of claim 125 or 126, said endosomal disruption agent being a fusogenic peptide.

128. A method of transfecting a mammalian cell with a recombinant nucleic acid, said method comprising the steps of

a) forming a mixture of a synthetic virus like particle as claimed in claim 1 and a neutral hydrophilic polymer, and

b) contacting said mixture of step a) with a mammalian cell under conditions sufficient to allow transfection of said cell with said nucleic acid.

129. A method of formulating a synthetic virus like particle for administration to a patient, the method comprising

mixing a synthetic virus like particle as claimed in claim 1 with a neutral hydrophilic polymer.

130. The method of claim 128 or 129, said neutral hydrophilic polymer comprising polyethyleneglycol.

131. A pharmaceutical formulation for administering a recombinant nucleic acid to a patient, comprising

the synthetic virus like particle of any one of claim 1 in combination with a pharmaceutically acceptable carrier.

132. The pharmaceutical formulation of claim 131, further comprising an endosomal disruption agent.

133. The pharmaceutical formulation of claim 131, further comprising a neutral hydrophilic polymer.

134. A method of introducing a recombinant nucleic acid into a patient, the method comprising

administering to the patient a therapeutically effective amount of the synthetic virus like particle of claim 1 or the pharmaceutical formulation of claim 131.

135. A method of making a synthetic virus like particle for transfecting mammalian cells with

a recombinant nucleic acid, said method comprising

mixing a recombinant nucleic acid with a plurality of nucleic acid condensing peptides under conditions sufficient to permit formation of a synthetic virus like particle containing condensed nucleic acid, wherein said nucleic acid condensing peptide is a heteropeptide and said plurality of nucleic acid condensing peptides has low polydispersion.

136. The method of claim 135 wherein said mixing step comprises mixing said nucleic acid with said plurality of nucleic acid condensing peptides wherein said plurality comprises a first nucleic acid condensing peptide and a second nucleic acid condensing peptide, each said nucleic acid condensing peptide being a heteropeptide and each said plurality of nucleic acid condensing peptides having low polydispersion, wherein said first nucleic acid condensing peptide comprises a first functional group covalently bound thereto.

137. A method of making a synthetic virus like particle for transfecting mammalian cells with a recombinant nucleic acid, said method comprising

mixing a recombinant nucleic acid with a first plurality of first nucleic acid condensing peptides and a second plurality of second nucleic acid condensing peptides under conditions sufficient to permit formation of a synthetic virus like particle containing condensed nucleic acid, wherein each said first and second nucleic acid condensing peptide is a heteropeptide and each said first and second plurality of nucleic acid condensing peptides has low polydispersion.

138. The method of claim 137 wherein said first nucleic acid condensing peptide comprises a first functional group covalently linked thereto.

139. The method of claim 136 or 137 wherein said first and said second nucleic acid condensing peptides have different amino acid sequences.

140. The method of claim 136 or 137, said first and said second nucleic acid condensing peptides having the same amino acid sequence.

141. The method of claim 136 or 137 wherein said mixing step comprises mixing said nucleic acid with a said plurality of nucleic acid condensing peptides wherein said second nucleic acid condensing peptide comprises a second functional group covalently bound thereto, said second functional group being different from said first functional group.

142. The method of claim 136 or 137 wherein said first nucleic acid condensing peptide further comprises a second functional group covalently bound thereto.

143. The method of claim 142 wherein said second functional group is covalently bound to said first functional group.

144. The method of claim 141 wherein said mixing step comprises the step of:

selecting a ratio of first and second functional groups such that the proportions of first and second nucleic acid condensing peptides mixed with said recombinant nucleic acid corresponds to said ratio.

145. The method of claim 141 wherein said mixing step comprises the step of:

selecting the position on a said nucleic acid condensing peptide for covalent linkage to a said functional group.

146. The method of claim 145, wherein said selecting step comprises selecting the amino acid position on said first nucleic acid condensing peptide for covalent linkage to said first functional group and selecting the amino acid position on said second nucleic acid condensing peptide for covalent linkage to said second functional group.

147. The method of claim 145 wherein said selecting step comprises selecting an amino or carboxy terminal position of a said nucleic acid condensing peptide.

148. The method of claim 141 wherein said mixing step comprises the step of :

selecting the position on a said functional group for covalent linkage to a said nucleic acid condensing peptide.

149. The method of claim 148 wherein said functional group comprises a targeting protein.

150. The method of claim 135 or 137, further comprising after said mixing step, the step of mixing said synthetic virus like particle with an endosomal disruption agent.

151. A method of making synthetic virus like particle for transfecting mammalian cells with a recombinant nucleic acid, said method comprising

a) contacting a plurality of first nucleic acid condensing peptides with a recombinant nucleic acid in high salt concentration for a time sufficient to allow noncovalent association of said plurality of nucleic acid condensing peptides with said nucleic acid and to permit condensation of said nucleic acid,

b) diluting said salt concentration of step a) to a lower salt concentration and adding a neutral hydrophilic polymer.

152. The method of claim 151 wherein in said step b), said diluting and adding is performed simultaneously with adding a plurality of second nucleic acid condensing peptides, each said second peptide comprising a lipid group covalently linked thereto,

wherein each said first and second nucleic acid condensing peptide is a heteropeptide and each said plurality has low polydispersion.

153. The method of claim 151 wherein said in said step b), said diluting and adding is performed prior to adding a plurality of second nucleic acid condensing peptides. each said second peptide comprising a lipid group covalently linked thereto,

wherein each said first and second nucleic acid condensing peptide is a heteropeptide and each said plurality has low polydispersion.

154. The method of claim 151 wherein said neutral hydrophilic polymer comprises polyethyleneglycol.

155. A method f making a synthetic virus like particle for transfecting mammalian cells with a recombinant nucleic acid, said method comprising

a) forming a mixture of a plurality of first nucleic acid condensing peptides and a recombinant nucleic acid in high salt concentration.

b) incubating said mixture of step a) for time sufficient to allow noncovalent association of said plurality of nucleic acid condensing peptides with said nucleic acid and condensation of said nucleic acid, and

c) contacting said mixture of step b) with a plurality of second nucleic acid condensing peptides comprising a lipid group covalently linked to a said second peptide,

wherein each said first and second nucleic acid condensing peptide is a heteropeptide and each said plurality of nucleic acid condensing peptides has low polydispersion.

FIG. 1(a)

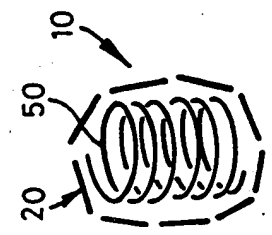


FIG. 1(b)

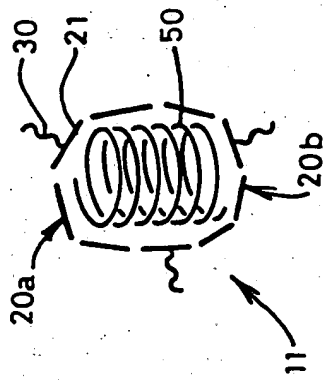


FIG. 1(c)

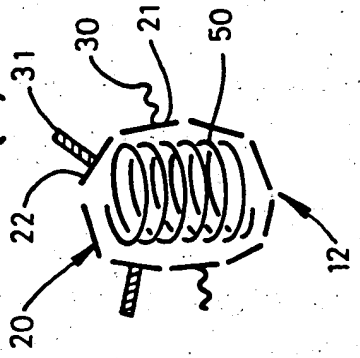


FIG. 1(d)

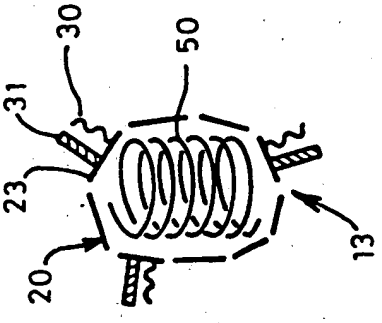


FIG. 1(e)

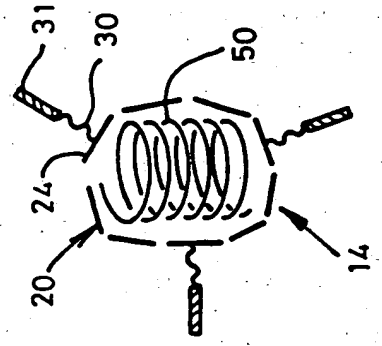
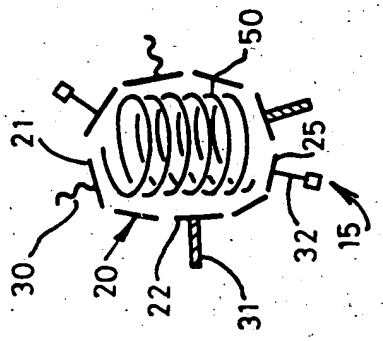
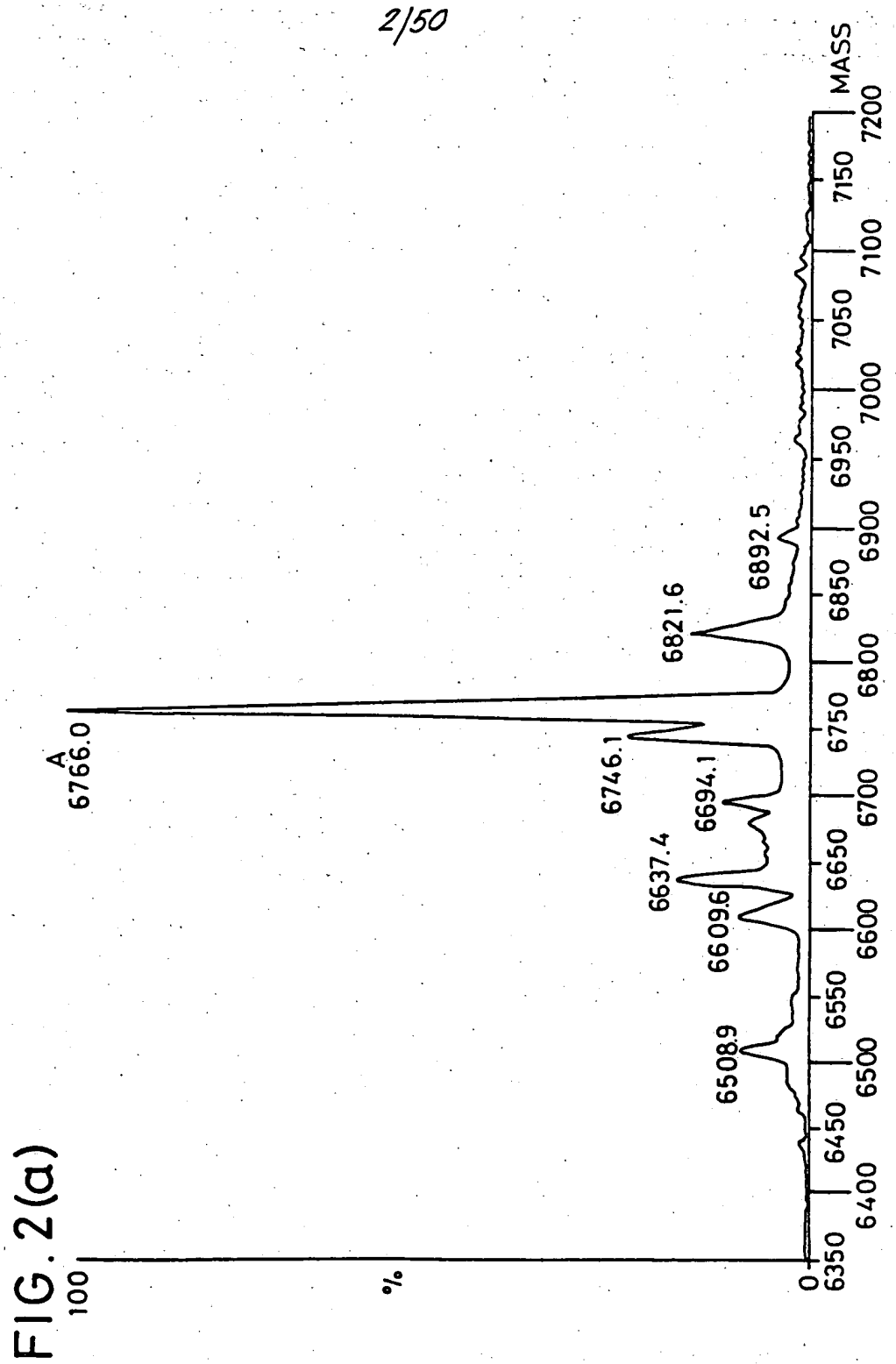


FIG. 1(f)



2/50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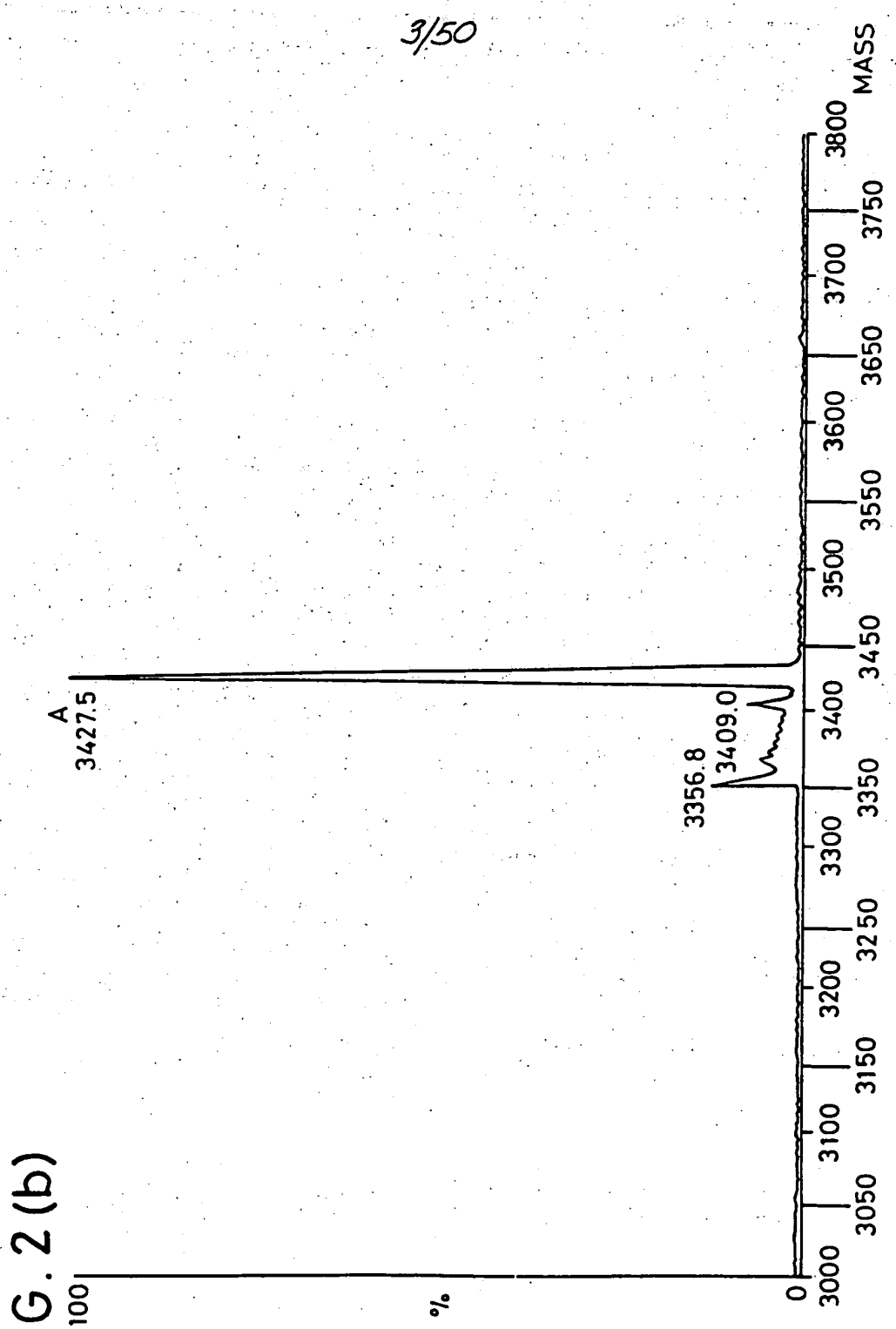
7150
7100
7050
7000
6950
6900
6850
6800
6750
6700
6650
6600
6550
6500
6450
6400
6350
100
%
0
A
6766.0
6746.1
6821.6
6892.5
6694.1
6637.4
6609.6
6508.9

FIG. 2(a)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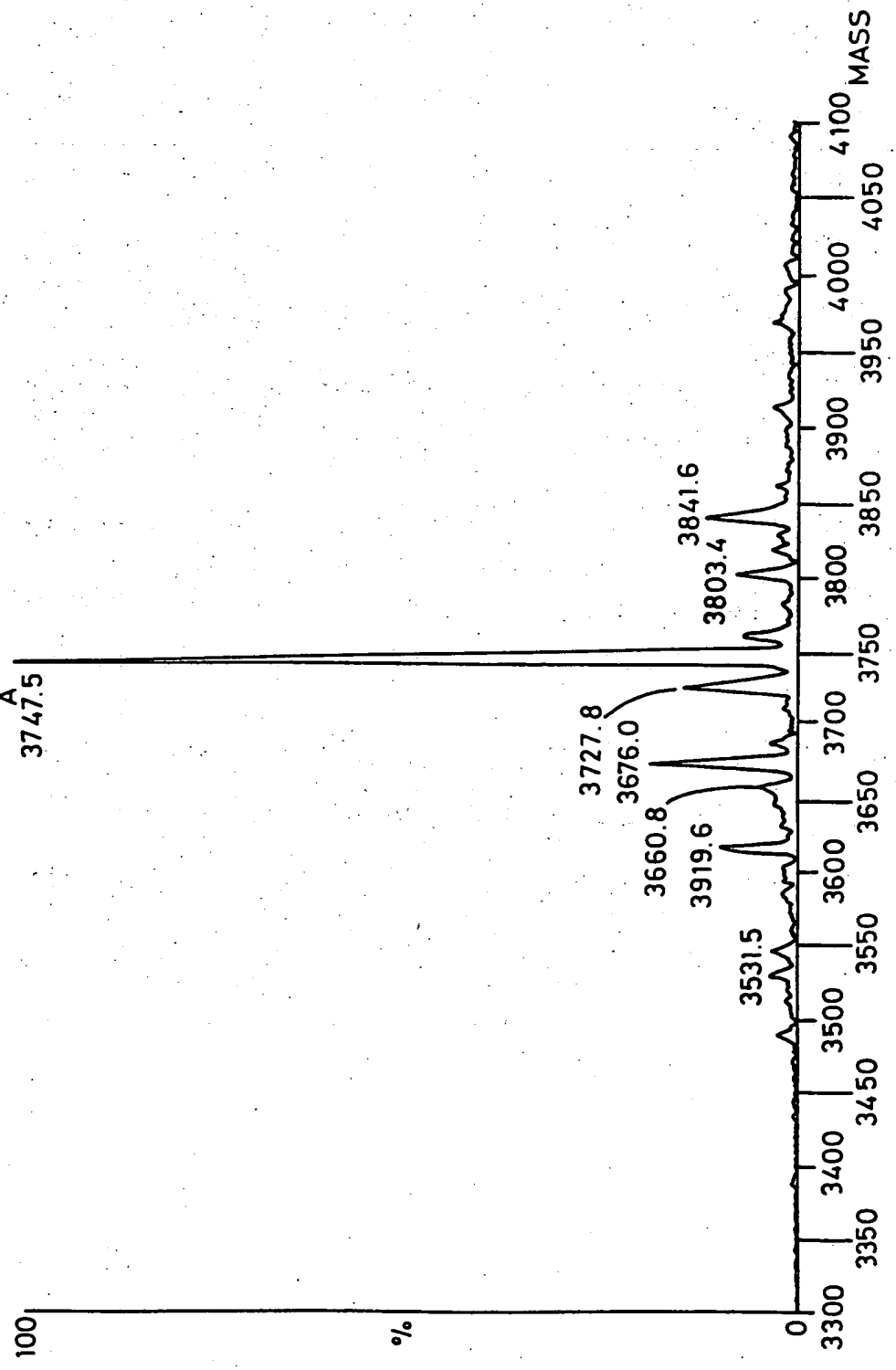
3050
3100
3150
3200
3250
3300
3350
3400
3450
3500
3550
3600
3650
3700
3750
3800
%
100
0
A
3427.5
3409.0
3356.8

FIG. 2 (b)


100
%
0
3300
3350
3400
3450
3500
3550
3600
3650
3700
3750
3800
3850
3900
3950
4000
4050
4100
MASS
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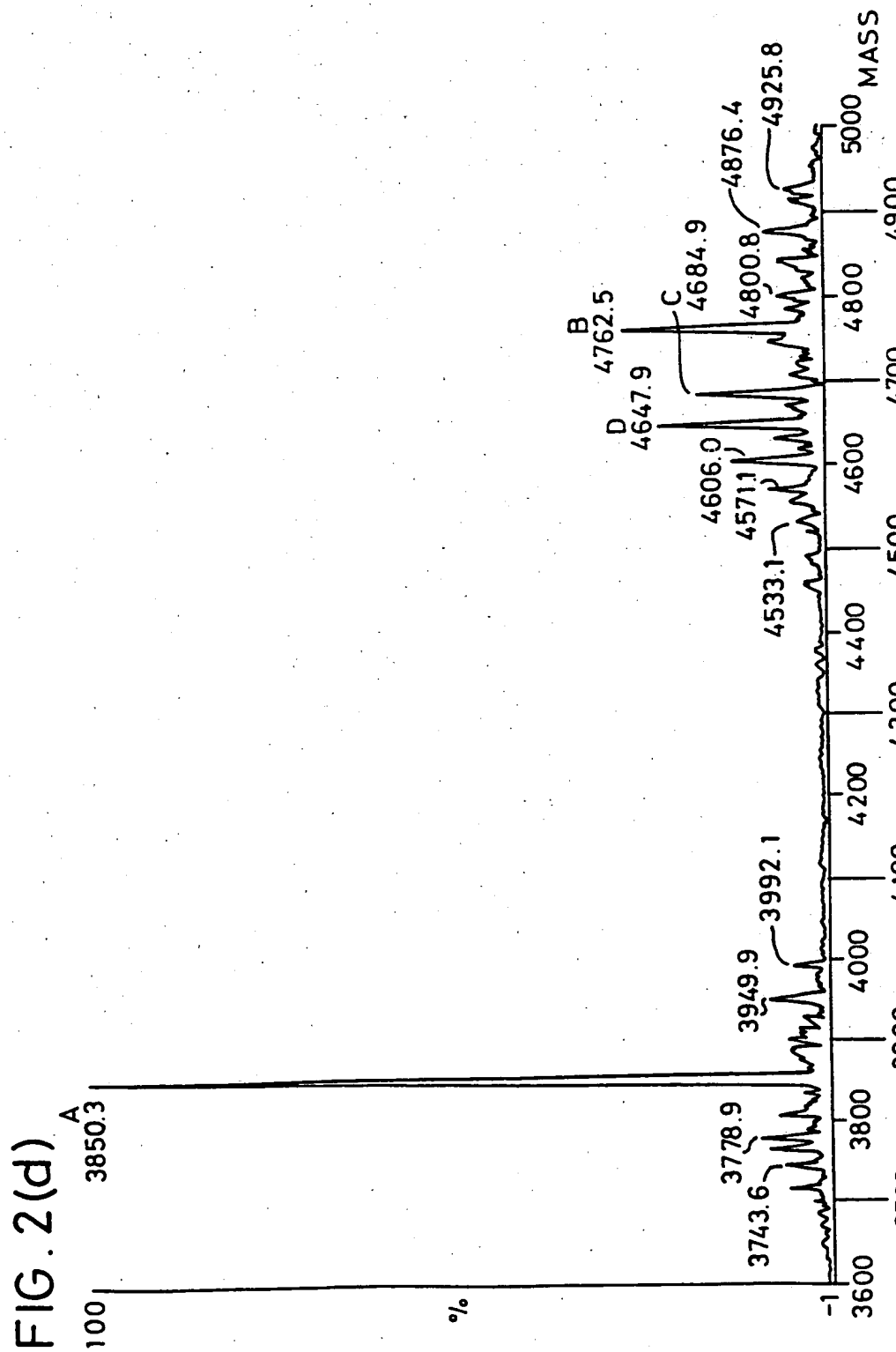
3727.8
3676.0
3660.8
3919.6
3531.5
3803.4
3841.6

FIG. 2(c)


100
%
-1
A
3850.3
3778.9
3743.6
3949.9
3992.1
B
4762.5
C
4684.9
D
4647.9
4606.0
4571.1
4533.1
4800.8
4876.4
4925.8
3600
3700
3800
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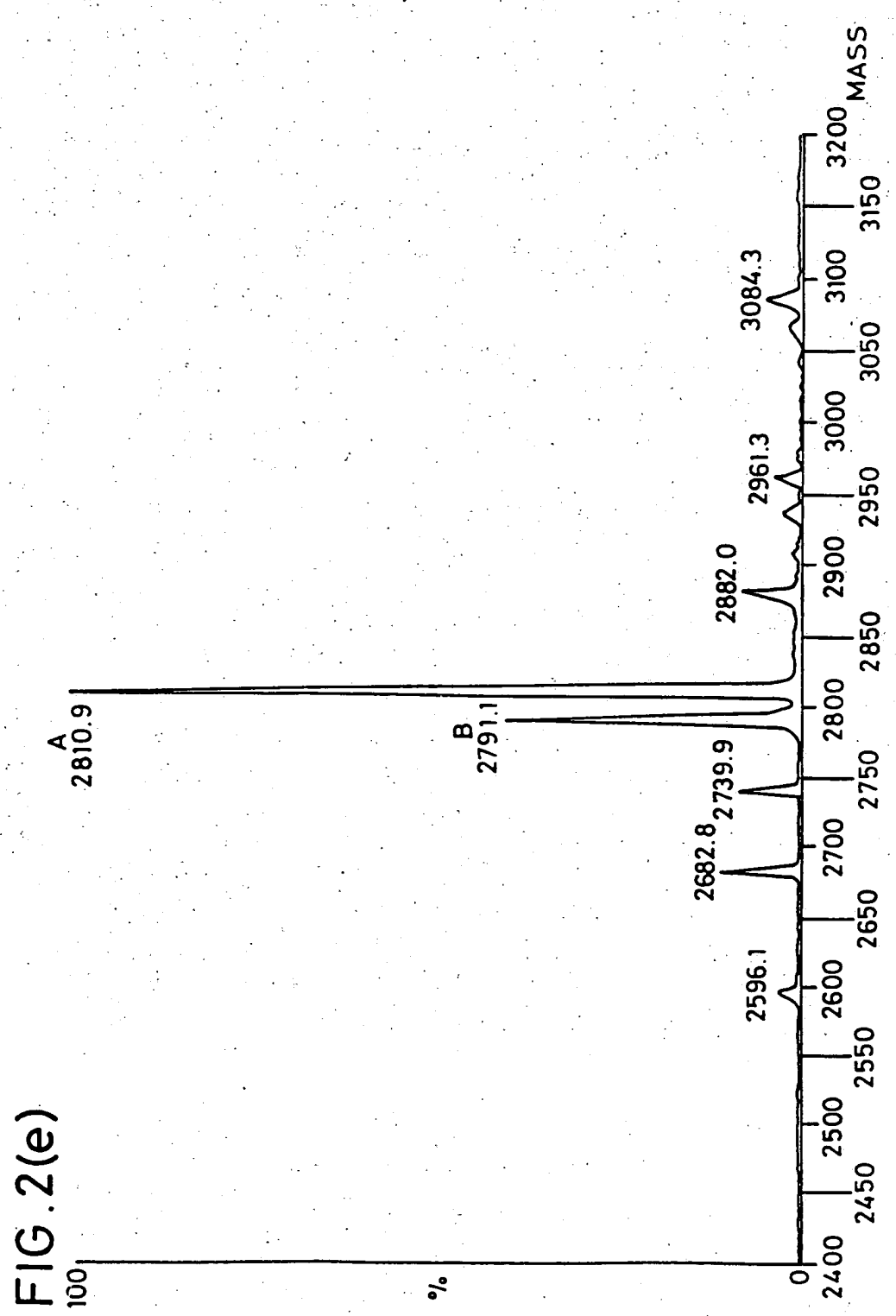
4400
4500
4600
4700
4800
4900
5000
MASS

FIG. 2(d)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%
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2450
2500
2550
2600
2650
2700
2750
2800
2850
2900
2950
3000
3050
3100
3150
3200
MASS
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B
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2739.9
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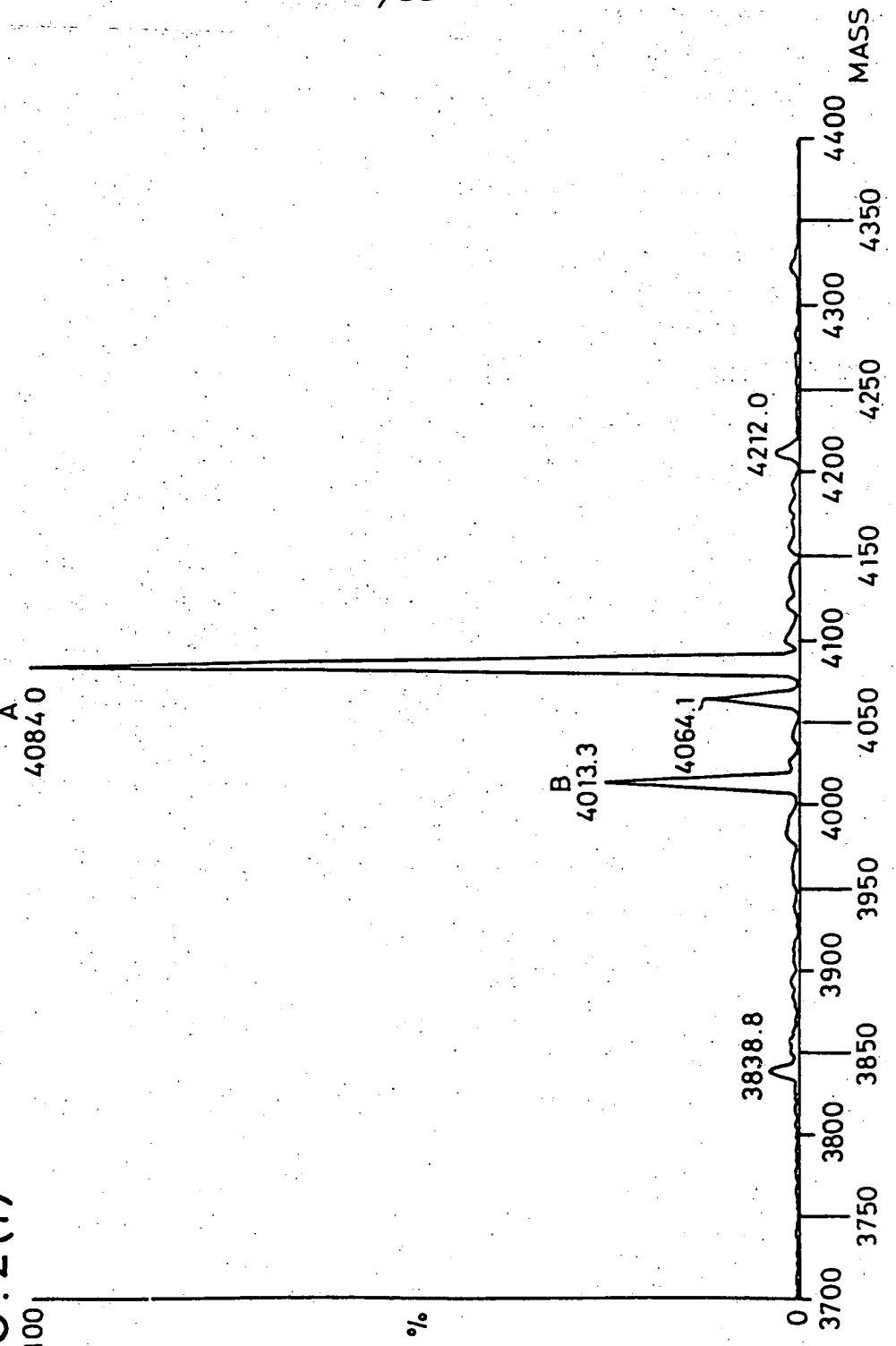
2882.0
2961.3
3084.3

FIG.2(e)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4300
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4200
4150
4100
4050
4000
3950
3900
3850
3800
3750
3700
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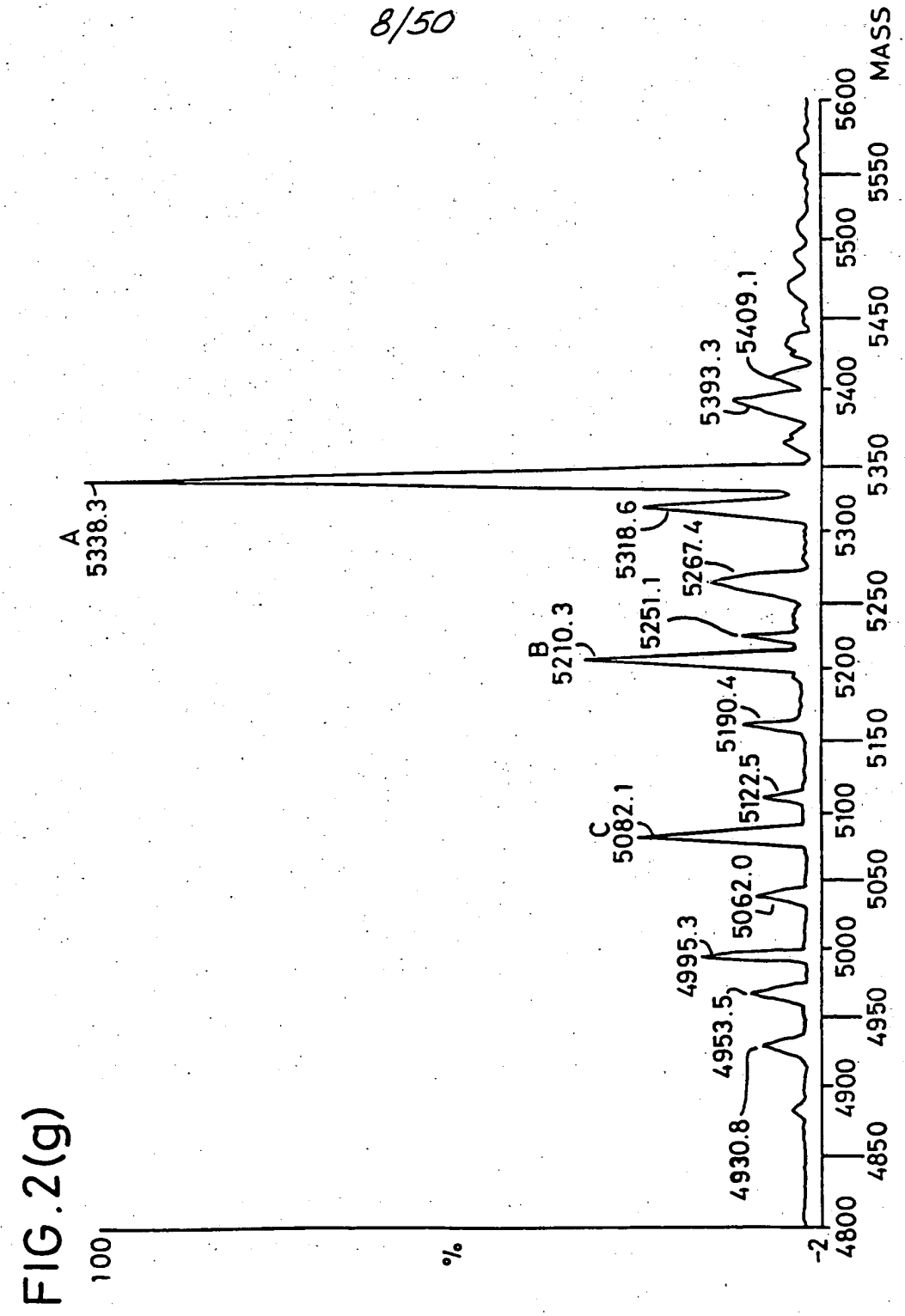
A
4084.0
4064.1
B
4013.3
3838.8
100
%
0

FIG. 2(f)


MASS
4800
4850
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4950
5000
5050
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5150
5200
5250
5300
5350
5400
5450
5500
5550
5600
%
100
-2
A
5338.3
B
5210.3
C
5082.1
4930.8
4953.5
4995.3
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5190.4
5251.1
5267.4
5318.6
5393.3
5409.1

FIG.2(g)

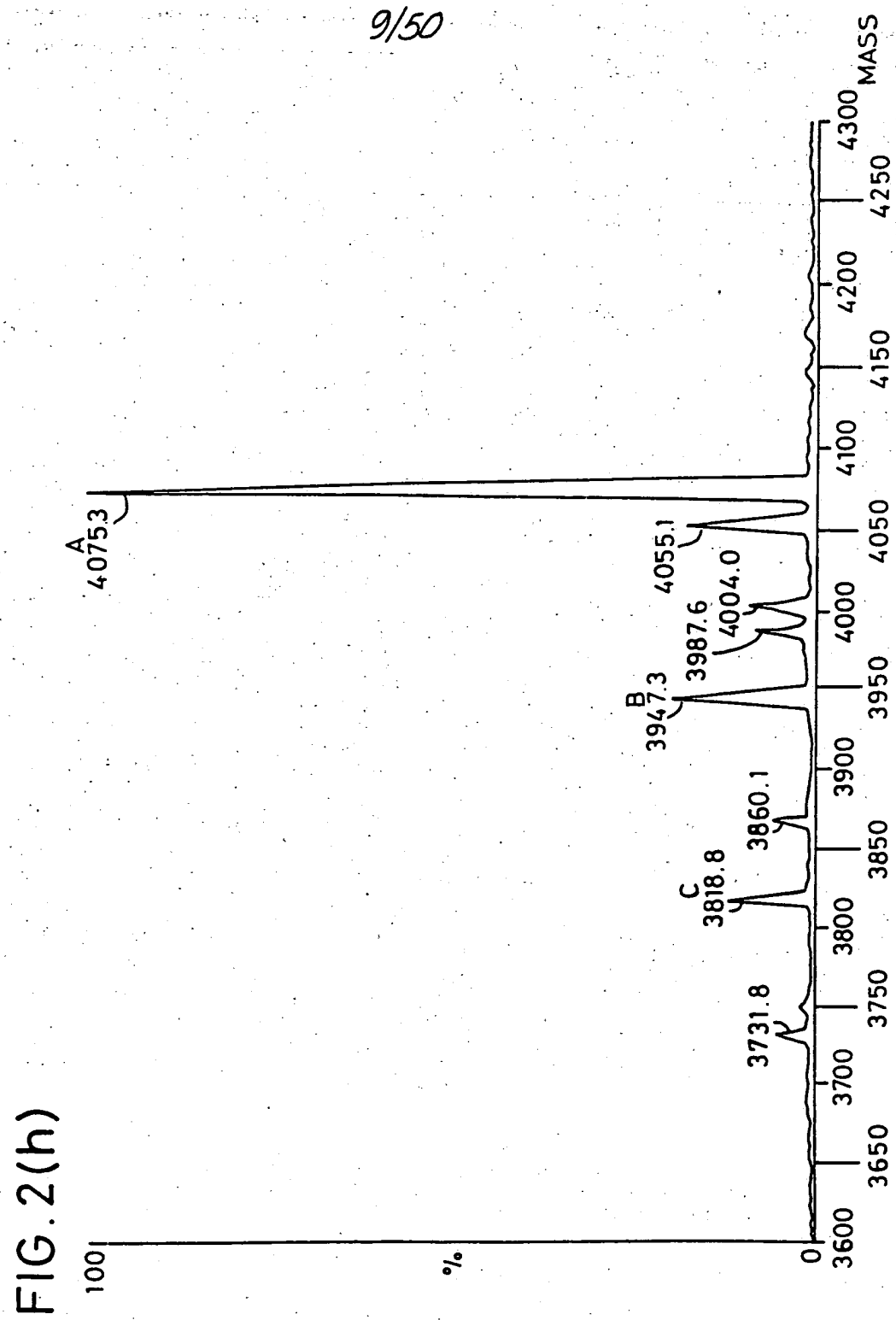

MASS
4300
4250
4200
4150
4100
4050
4000
3950
3900
3850
3800
3750
3700
3650
3600
A
4075.3
4055.1
4004.0
3987.6
B
3947.3
3860.1
C
3818.8
3731.8
100
%
0

FIG. 2(h)

INCREASING PEPTIDE TO DNA MASS RATIO

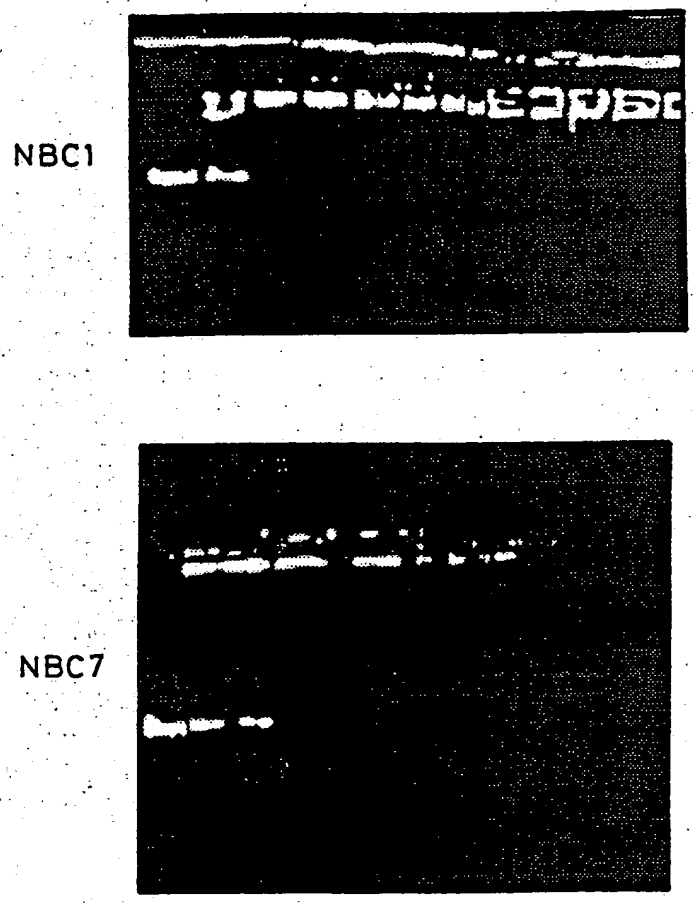
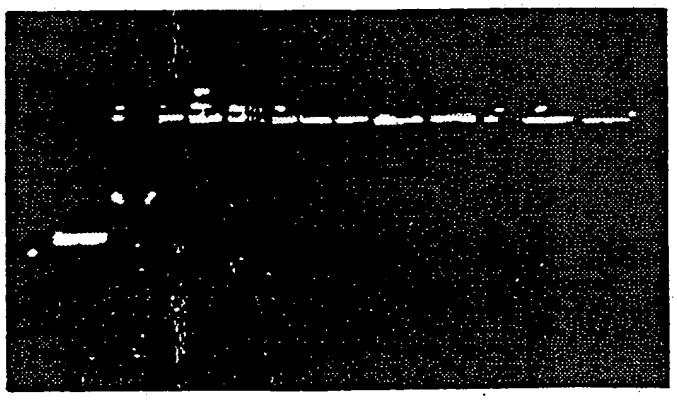


FIG.3



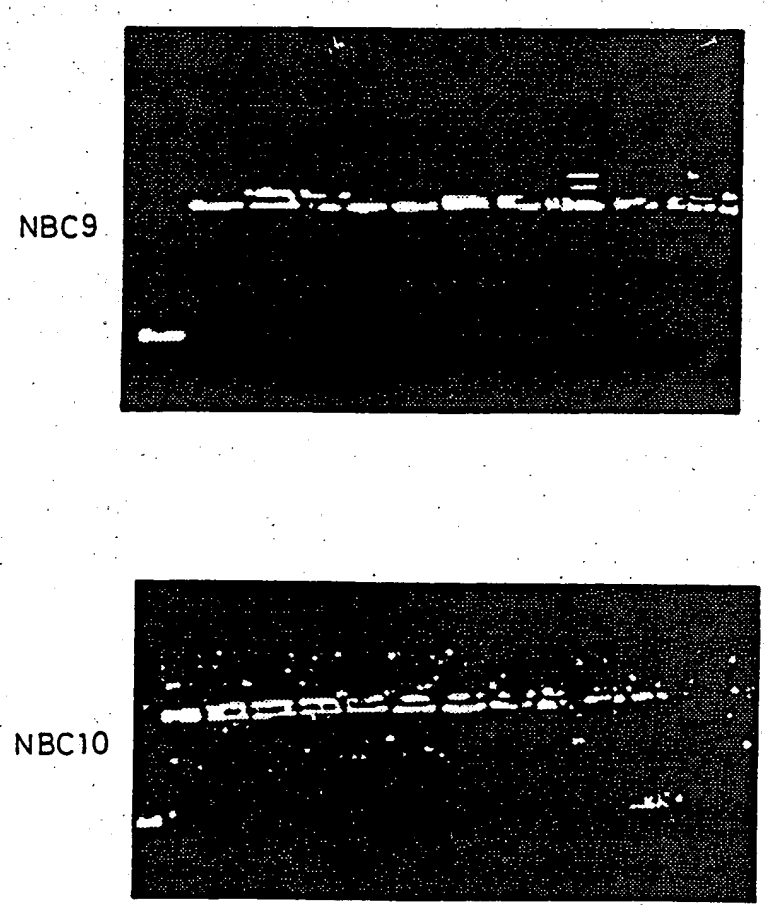


FIG. 3 (CONTD.)

FIG. 4(a)

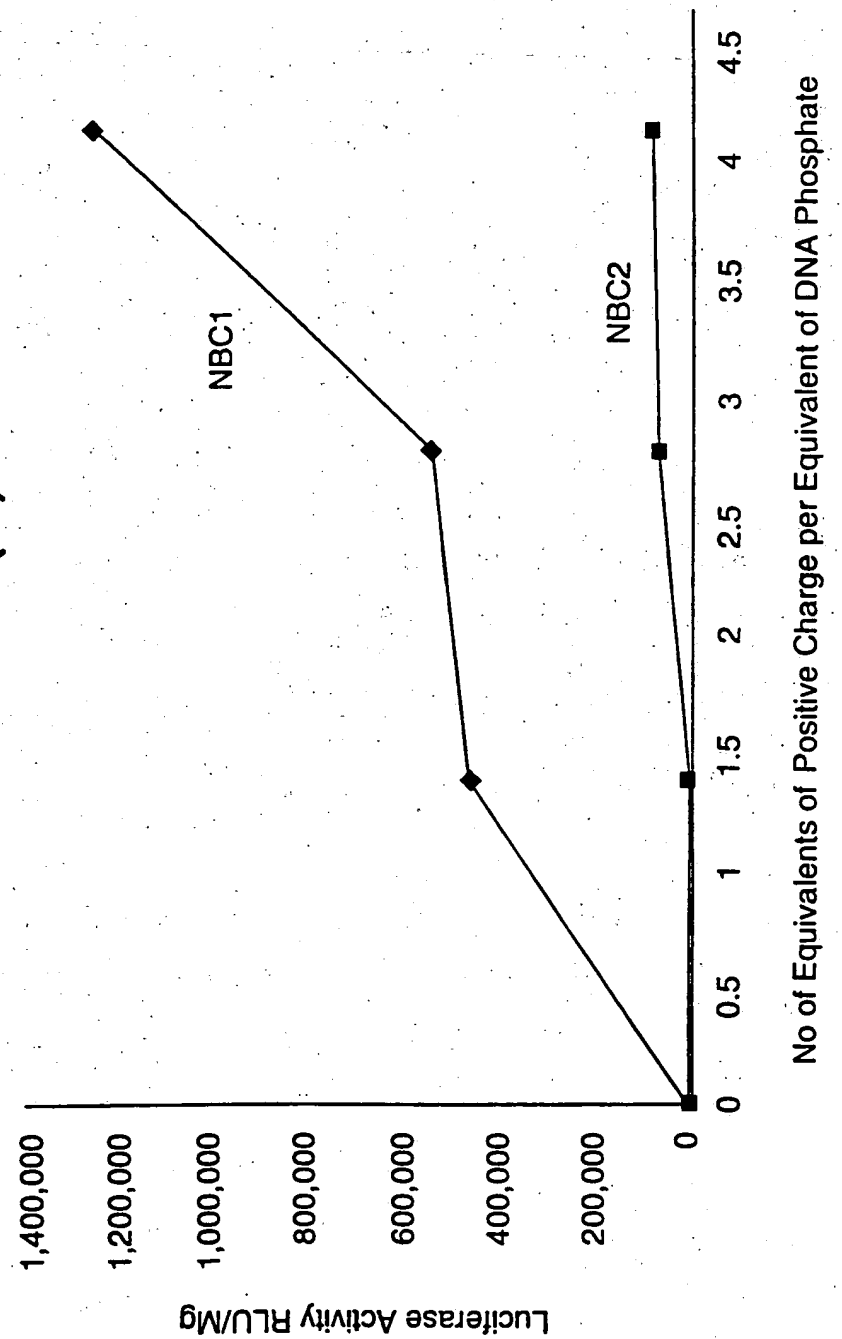
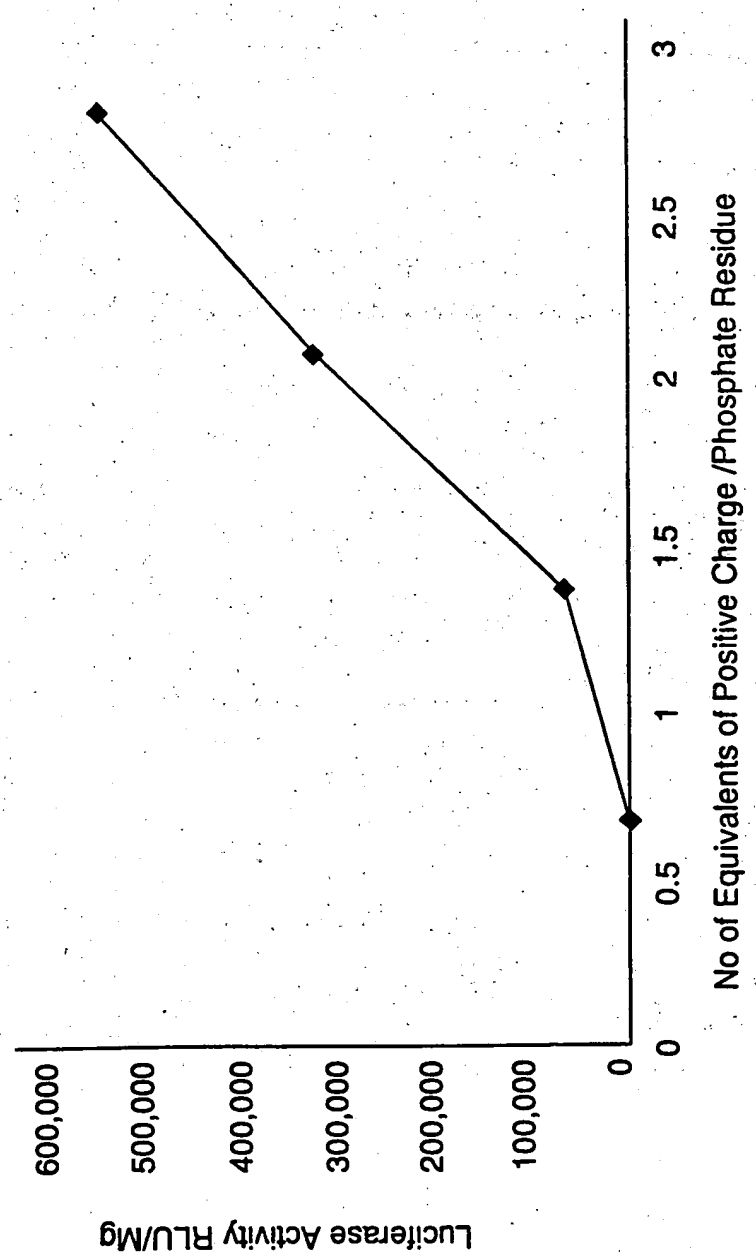

NBC1
NBC2
Luciferase Activity RLU/Mg
1,400,000
1,200,000
1,000,000
800,000
600,000
400,000
200,000
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
No of Equivalents of Positive Charge per Equivalent of DNA Phosphate

FIG. 4(b)


Luciferase Activity RLU/Mg
600,000
500,000
400,000
300,000
200,000
100,000
0
0
0.5
1
1.5
2
2.5
3
No of Equivalents of Positive Charge /Phosphate Residue

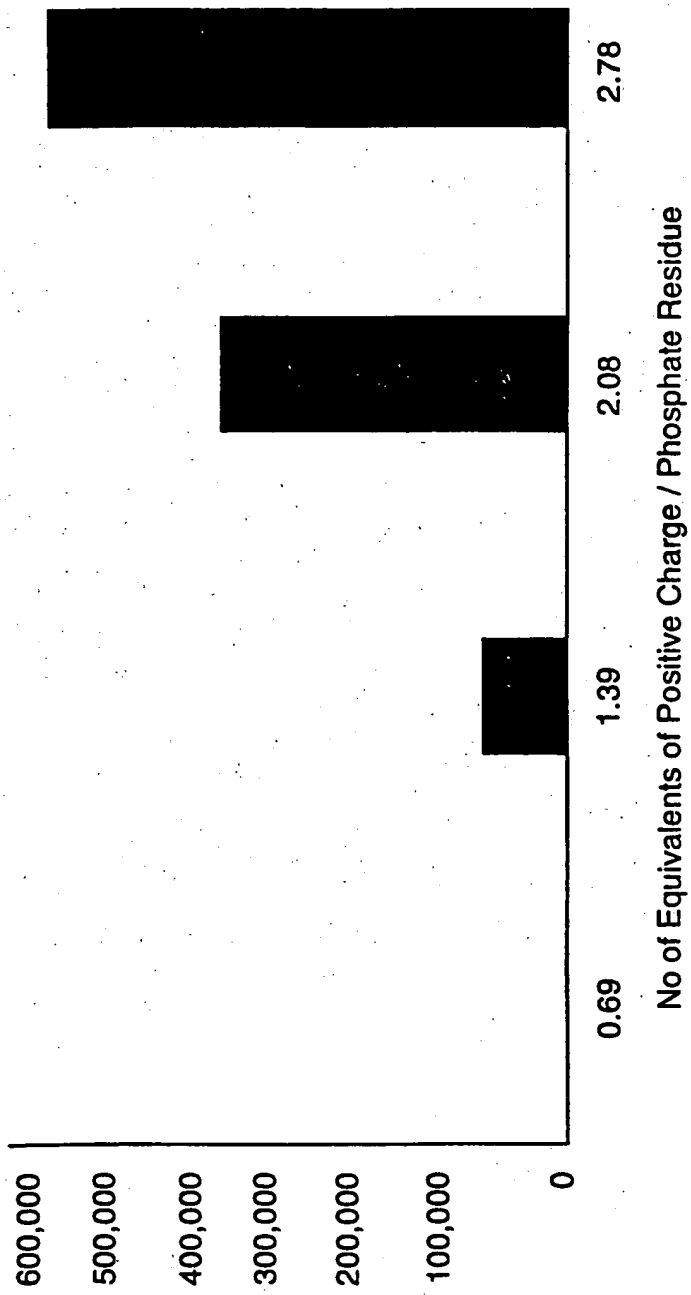

2.78
2.08
1.39
0.69
No of Equivalents of Positive Charge / Phosphate Residue
600,000
500,000
400,000
300,000
200,000
100,000
0

FIG. 4(c)

FIG. 5

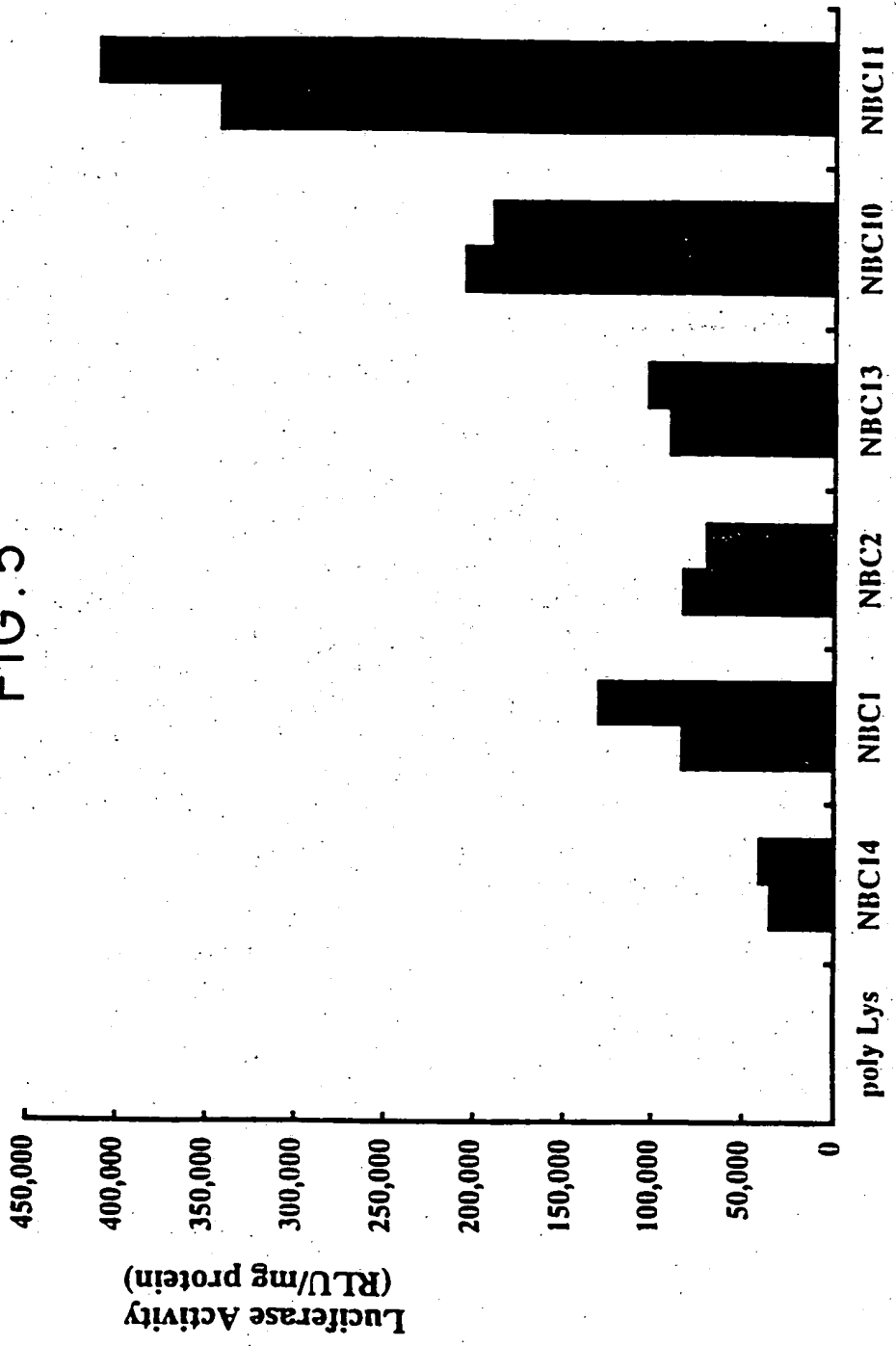
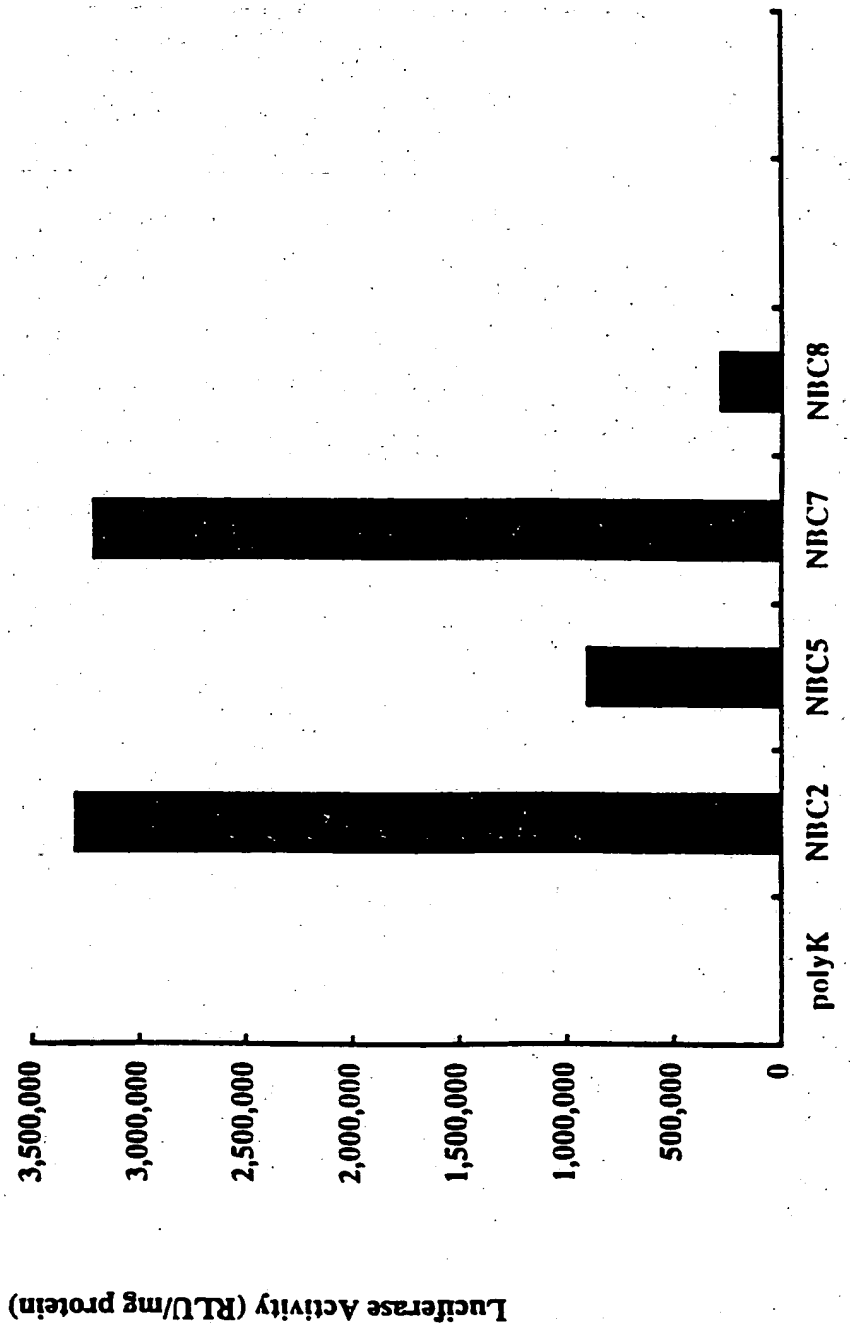

Luciferase Activity
(RLU/mg protein)
450,000
400,000
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
poly Lys
NBC14
NBC1
NBC2
NBC13
NBC10
NBC11

FIG. 6(a)


Luciferase Activity (RLU/mg protein)
3,500,000
3,000,000
2,500,000
2,000,000
1,500,000
1,000,000
500,000
0
polyK
NBC2
NBC5
NBC7
NBC8

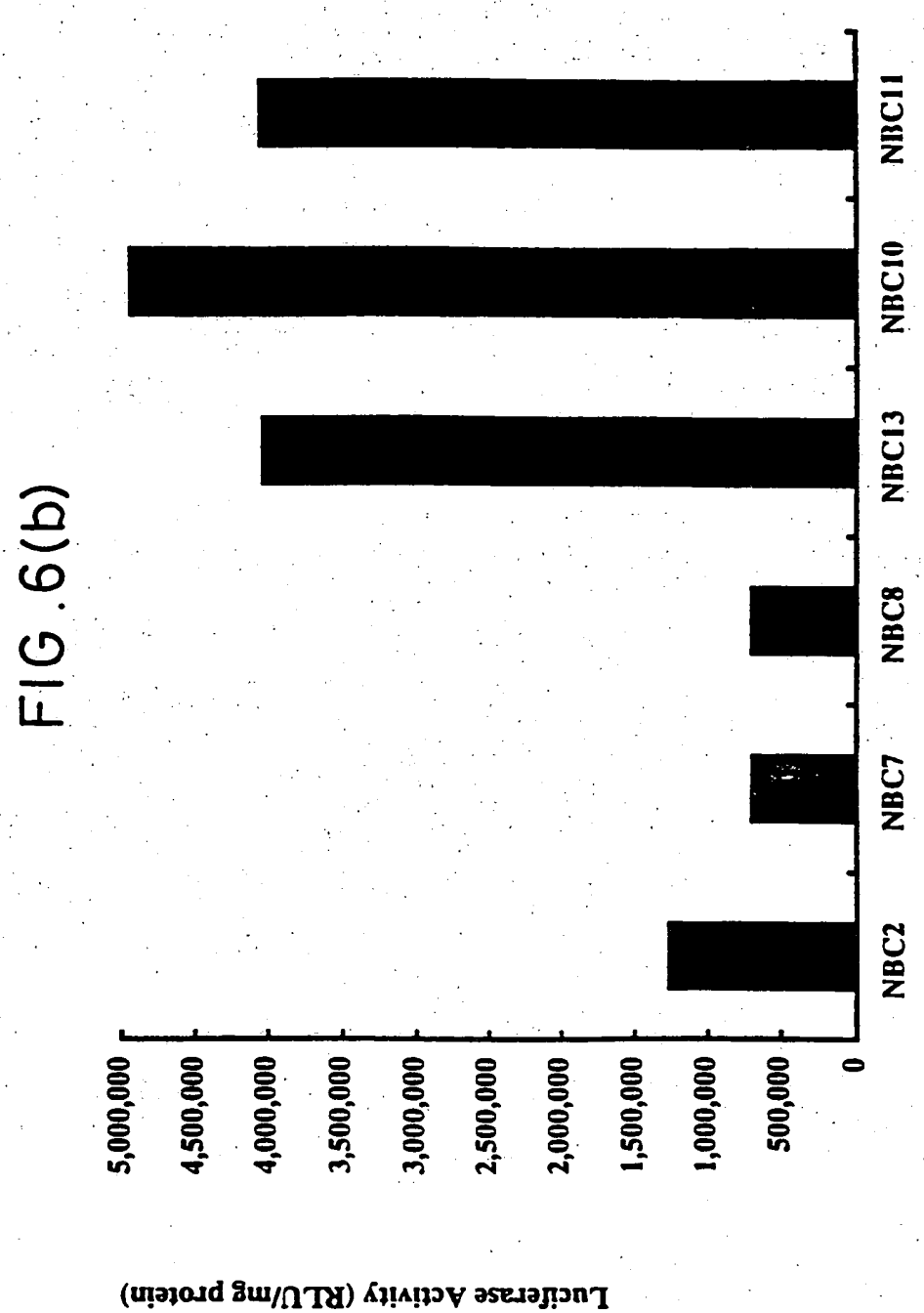

NBC11
NBC10
NBC13
NBC8
NBC7
NBC2
0
500,000
1,000,000
1,500,000
2,000,000
2,500,000
3,000,000
3,500,000
4,000,000
4,500,000
5,000,000
Luciferase Activity (RLU/mg protein)

FIG.6(b)

FIG. 7

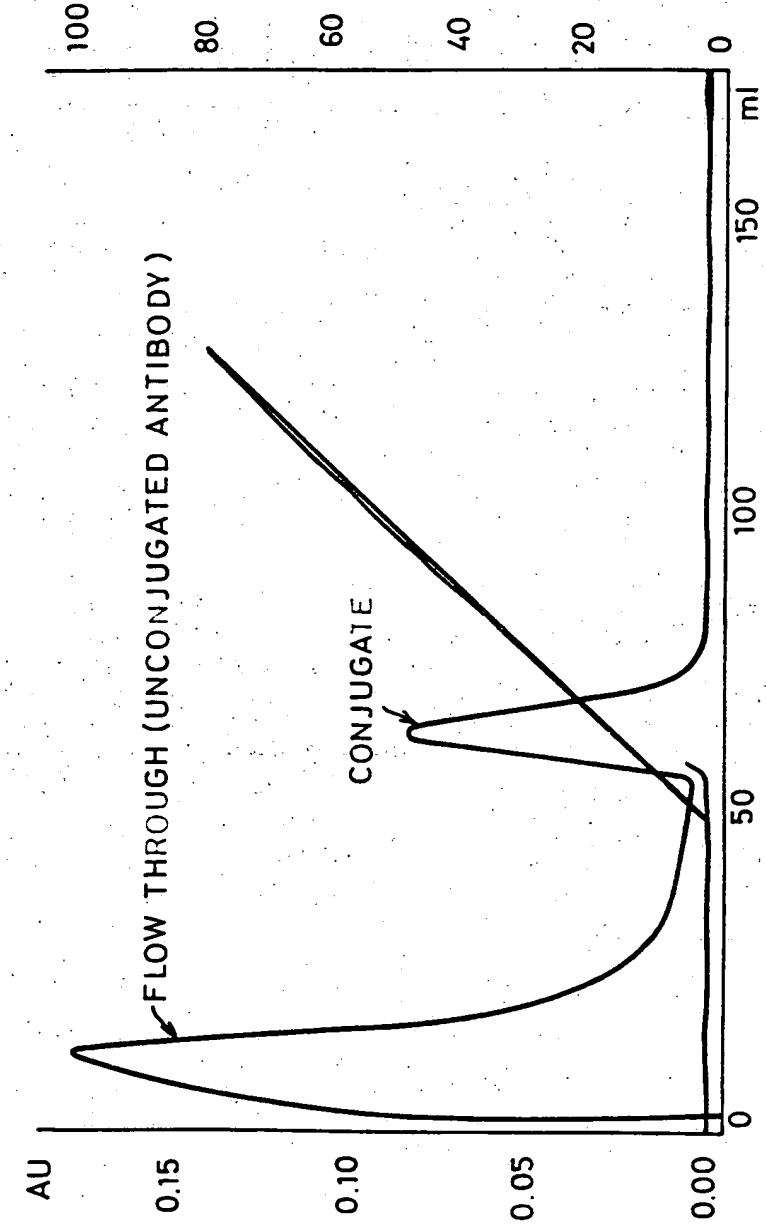
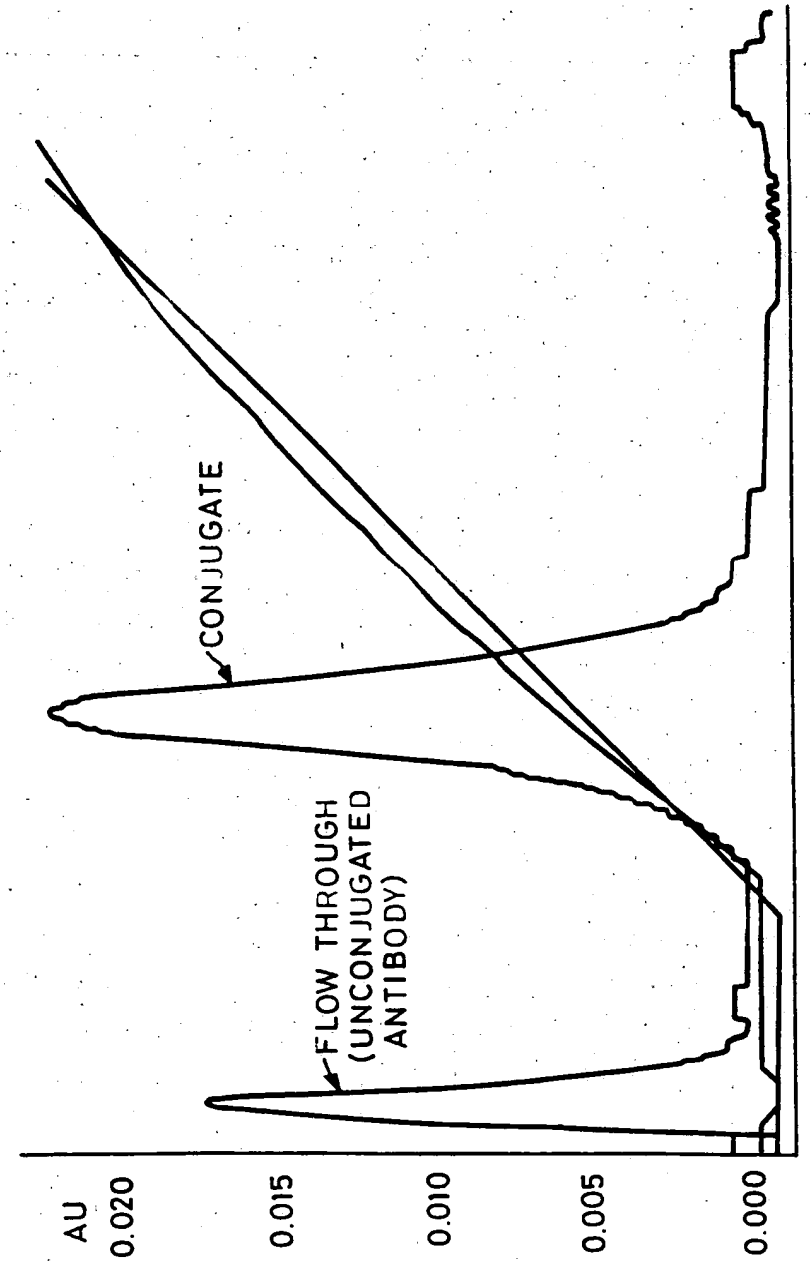

AU
0.15
0.10
0.05
0.00
0
50
100
150
ml
0
20
40
60
80
100
FLOW THROUGH (UNCONJUGATED ANTIBODY)
CONJUGATE

FIG. 8


CONJUGATE
FLOW THROUGH (UNCONJUGATED ANTIBODY)
AU
0.020
0.015
0.010
0.005
0.000

20/50

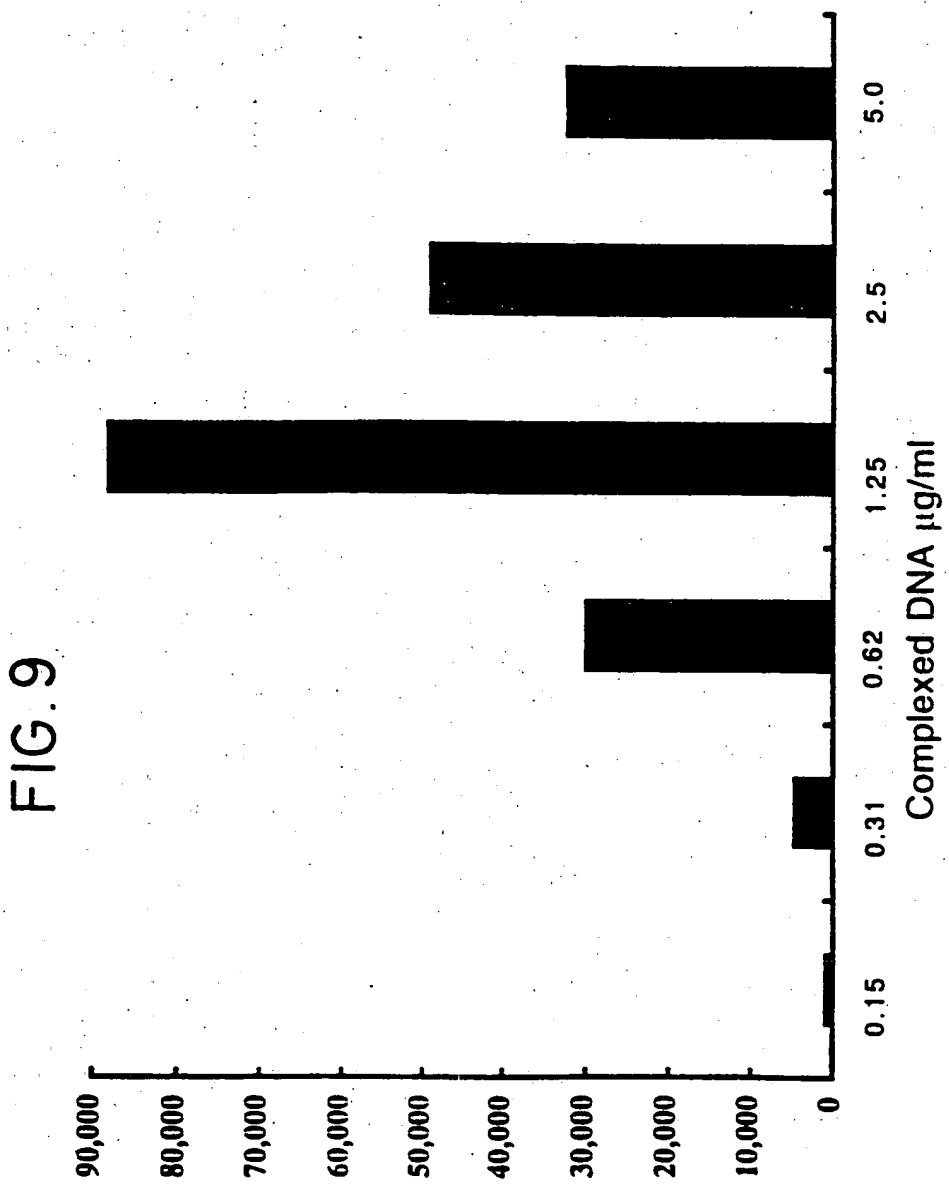

90,000
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
0.15
0.31
0.62
1.25
2.5
5.0
Complexed DNA µg/ml
Luciferase Counts/mg
Luciferase Activity (RLU/mg protein)

FIG. 9

FIG. 10

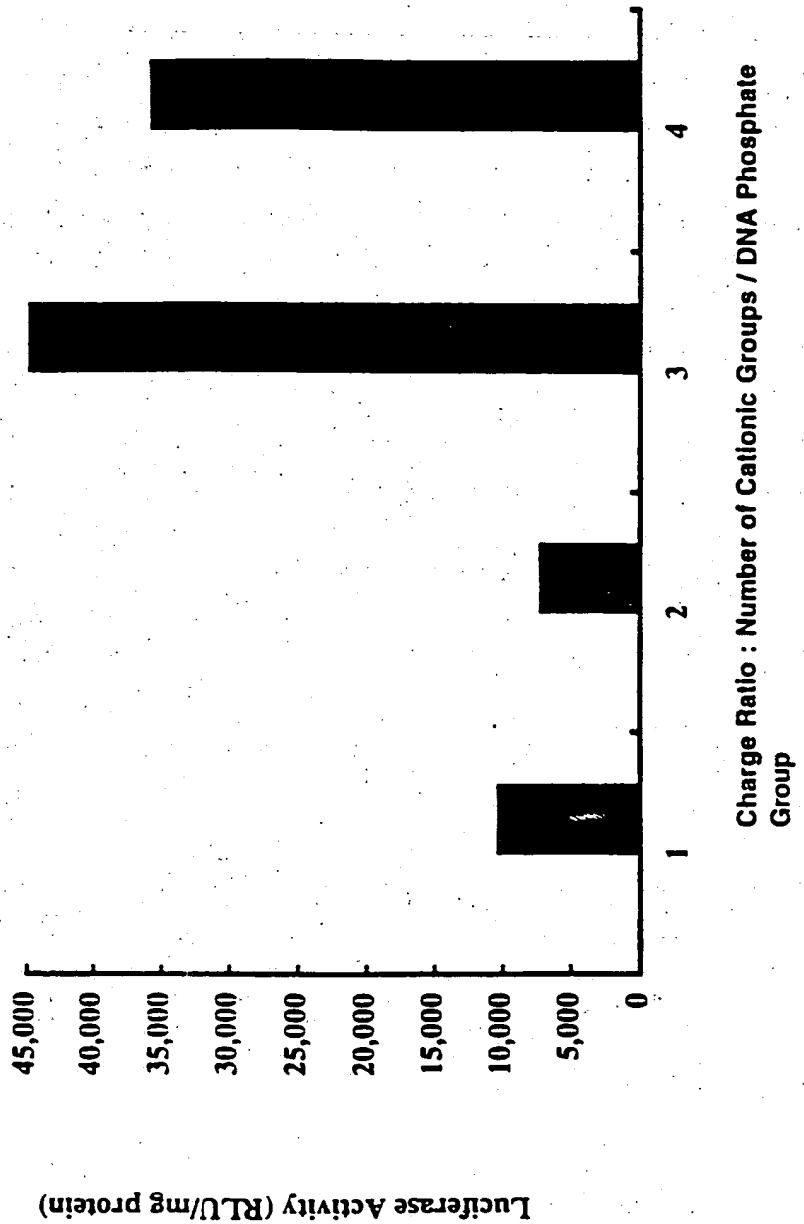

Luciferase Activity (RLU/mg protein)
45,000
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
1
2
3
4
Charge Ratio : Number of Cationic Groups / DNA Phosphate Group

FIG. 11

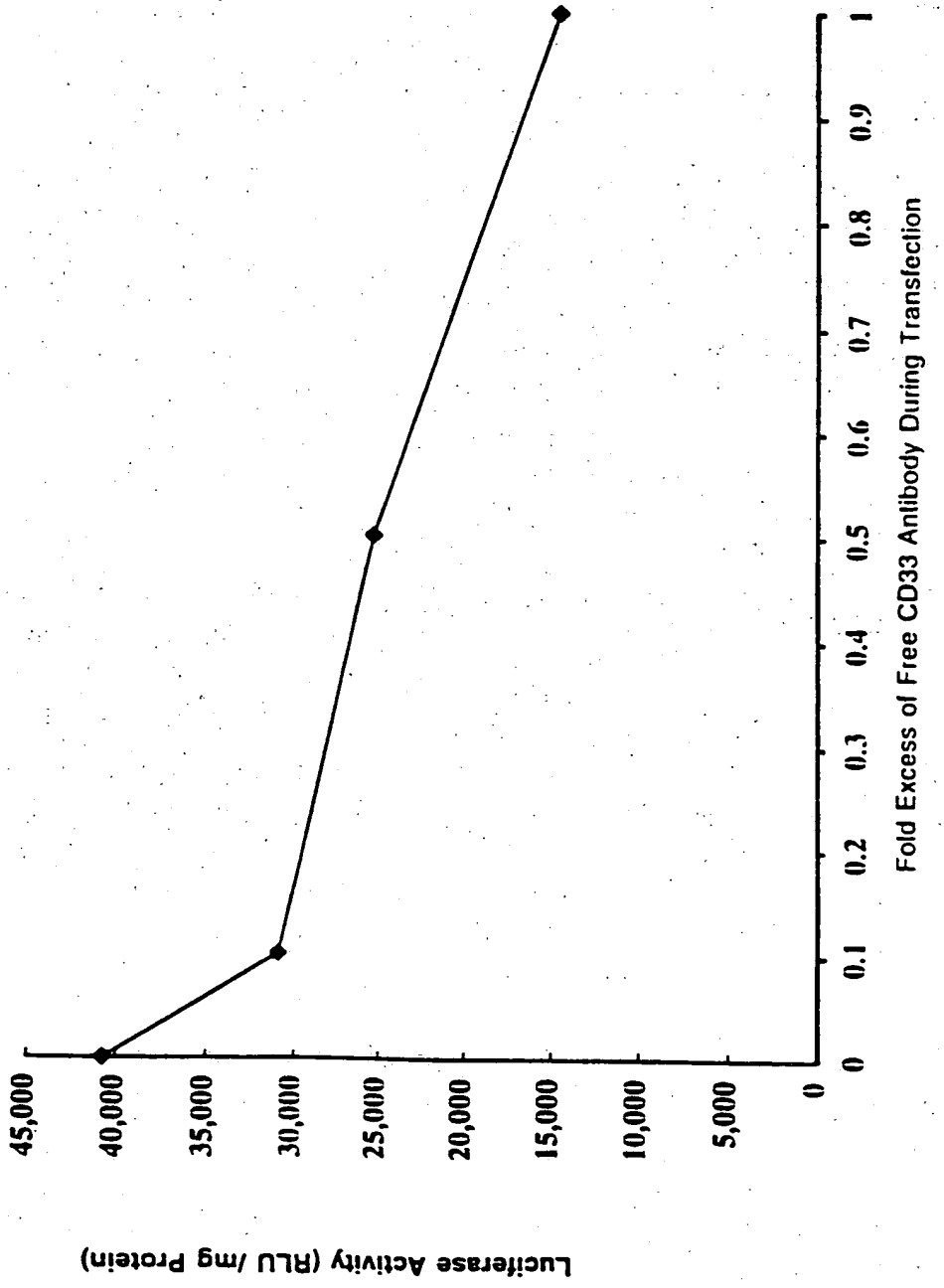

Luciferase Activity (RLU /mg Protein)
45,000
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Fold Excess of Free CD33 Antibody During Transfection

FIG. 12

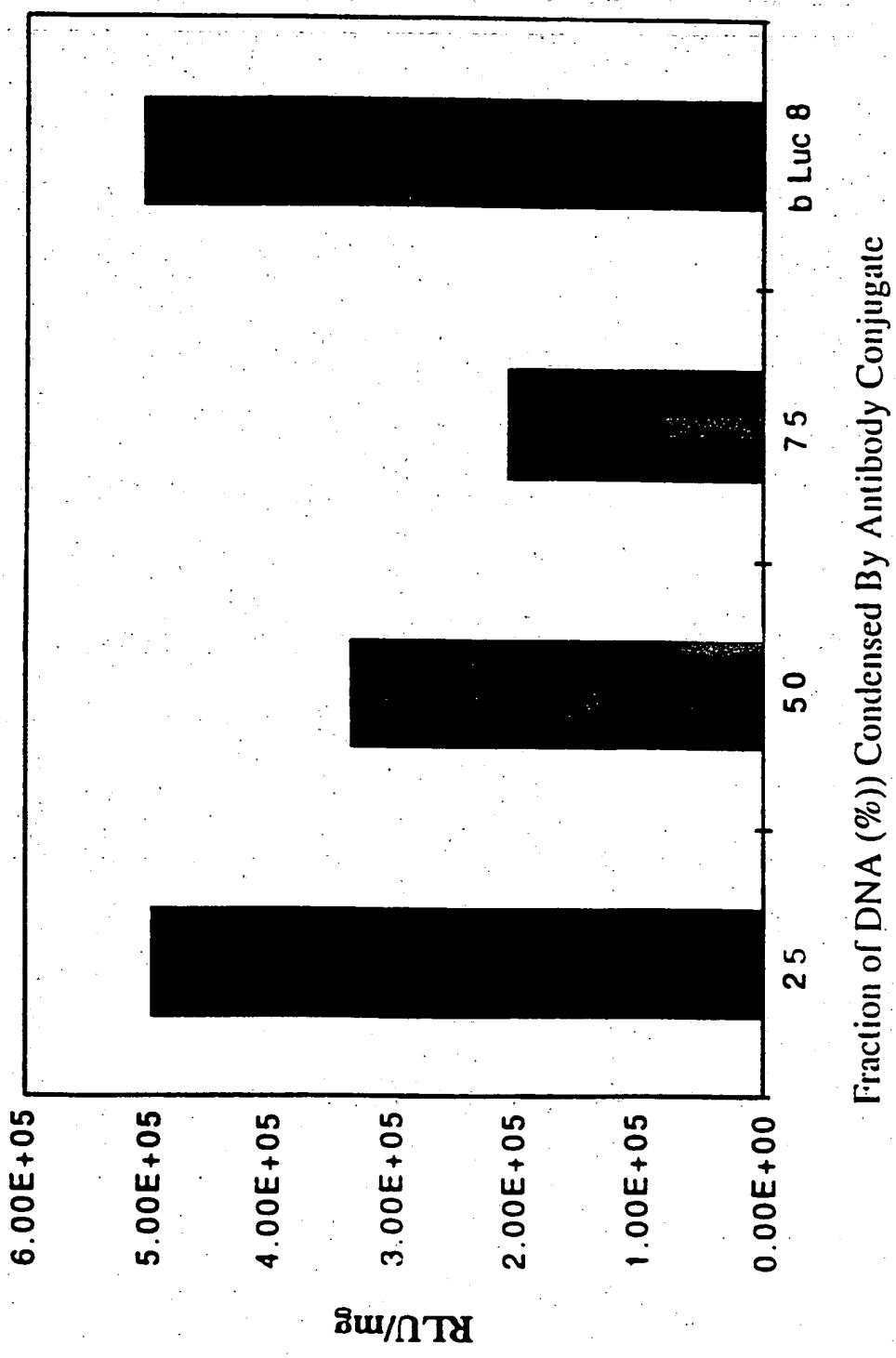

RLU/mg
6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
25
50
75
b Luc 8
Fraction of DNA (%)) Condensed By Antibody Conjugate

FIG. 13

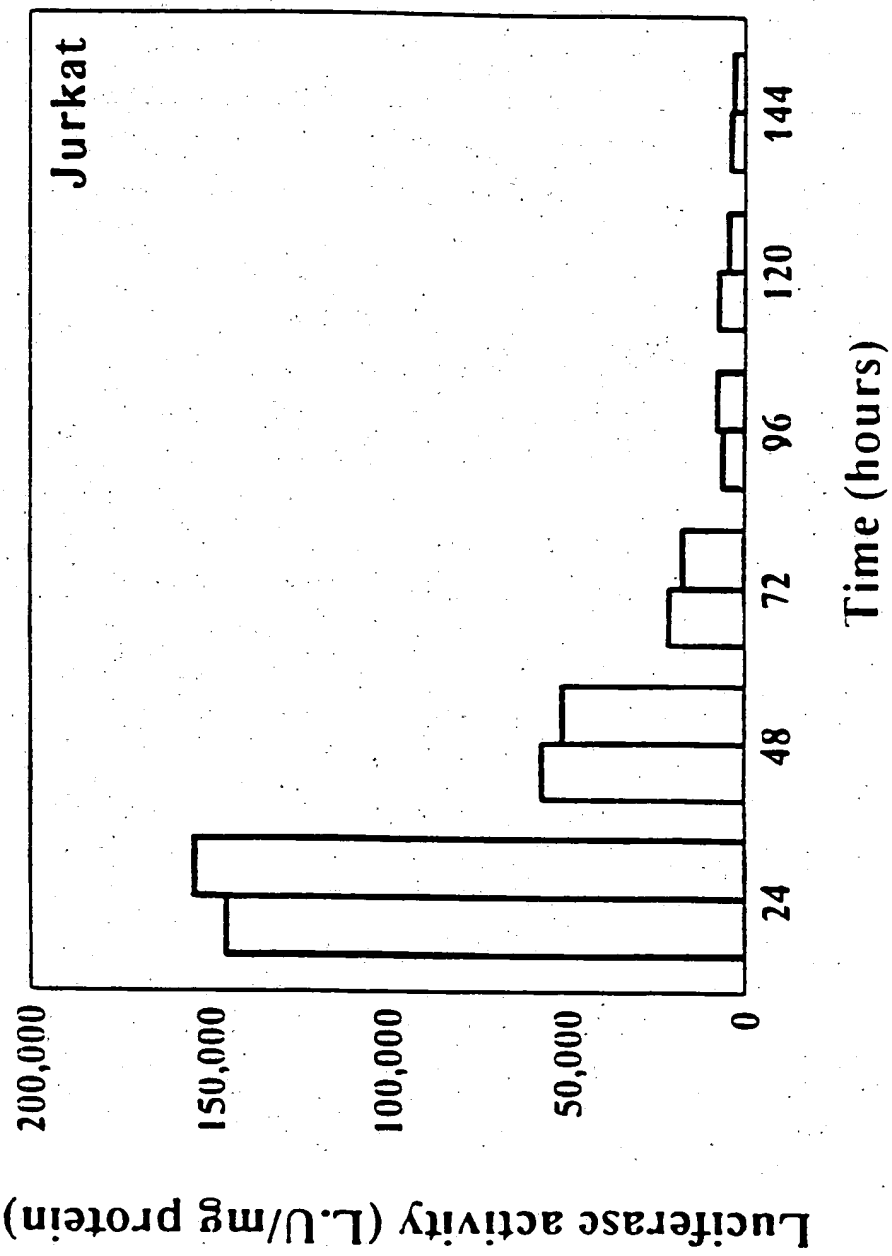

Jurkat
Luciferase activity (L.U/mg protein)
200,000
150,000
100,000
50,000
0
24
48
72
96
120
144
Time (hours)

FIG. 14

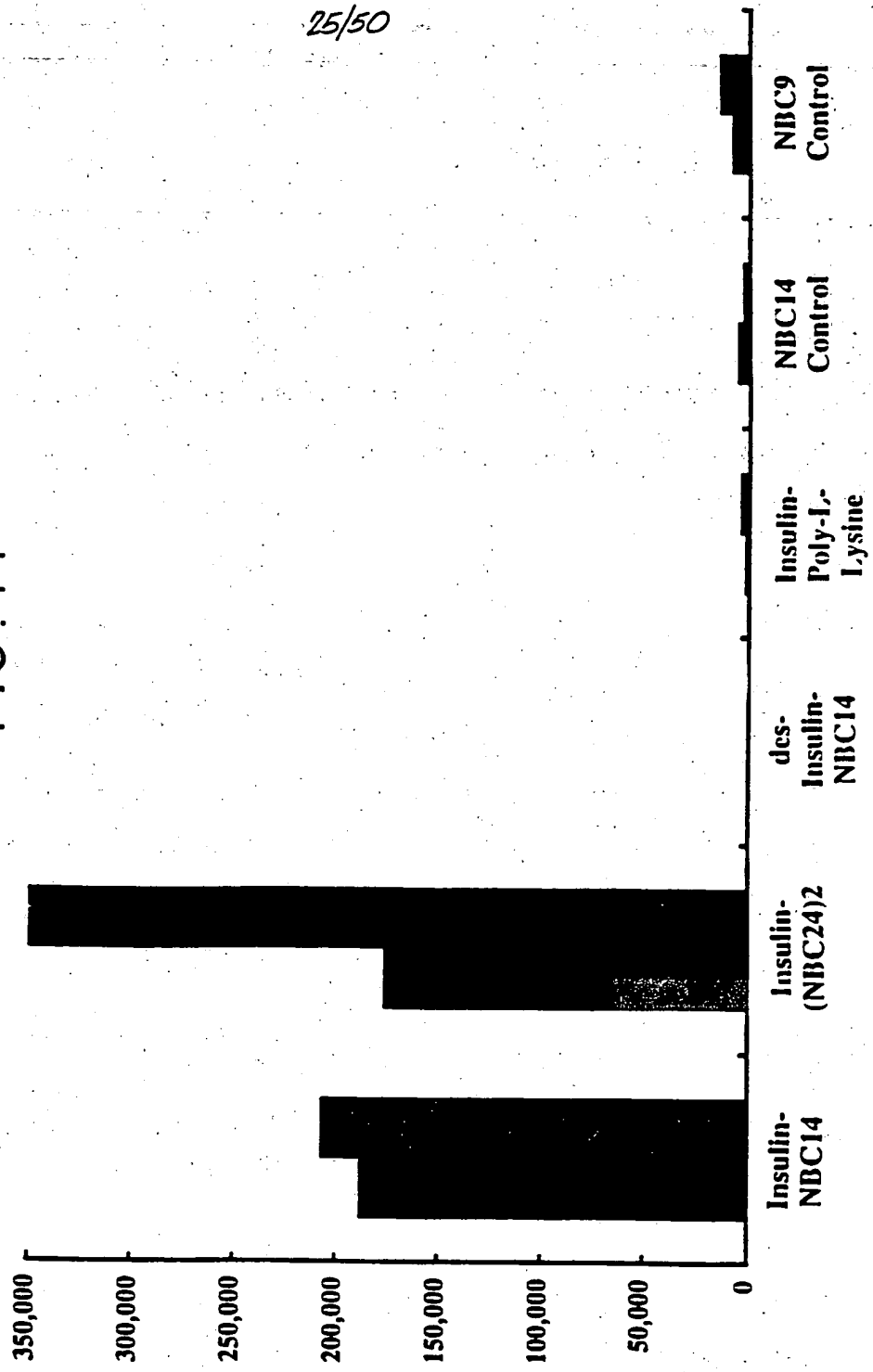

Luciferase Activity
(RLU/mg protein)
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
Insulin-NBC14
Insulin-(NBC24)2
des-Insulin-NBC14
Insulin-Poly-L-Lysine
NBC14 Control
NBC9 Control

FIG. 15

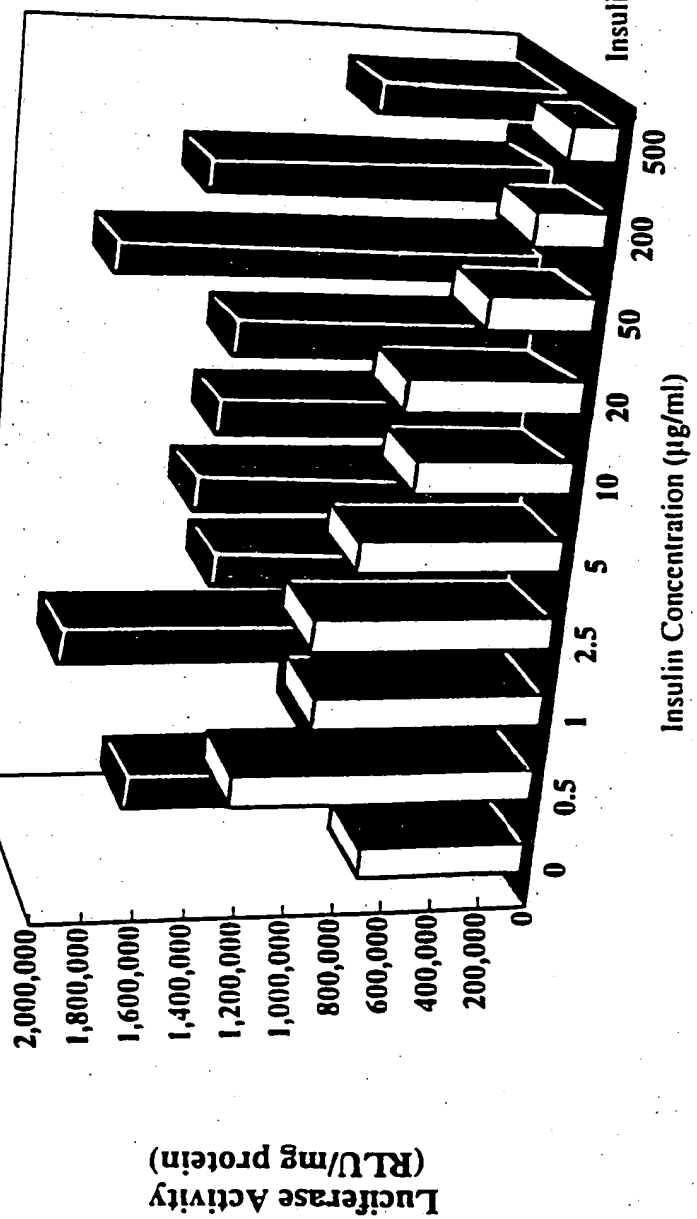
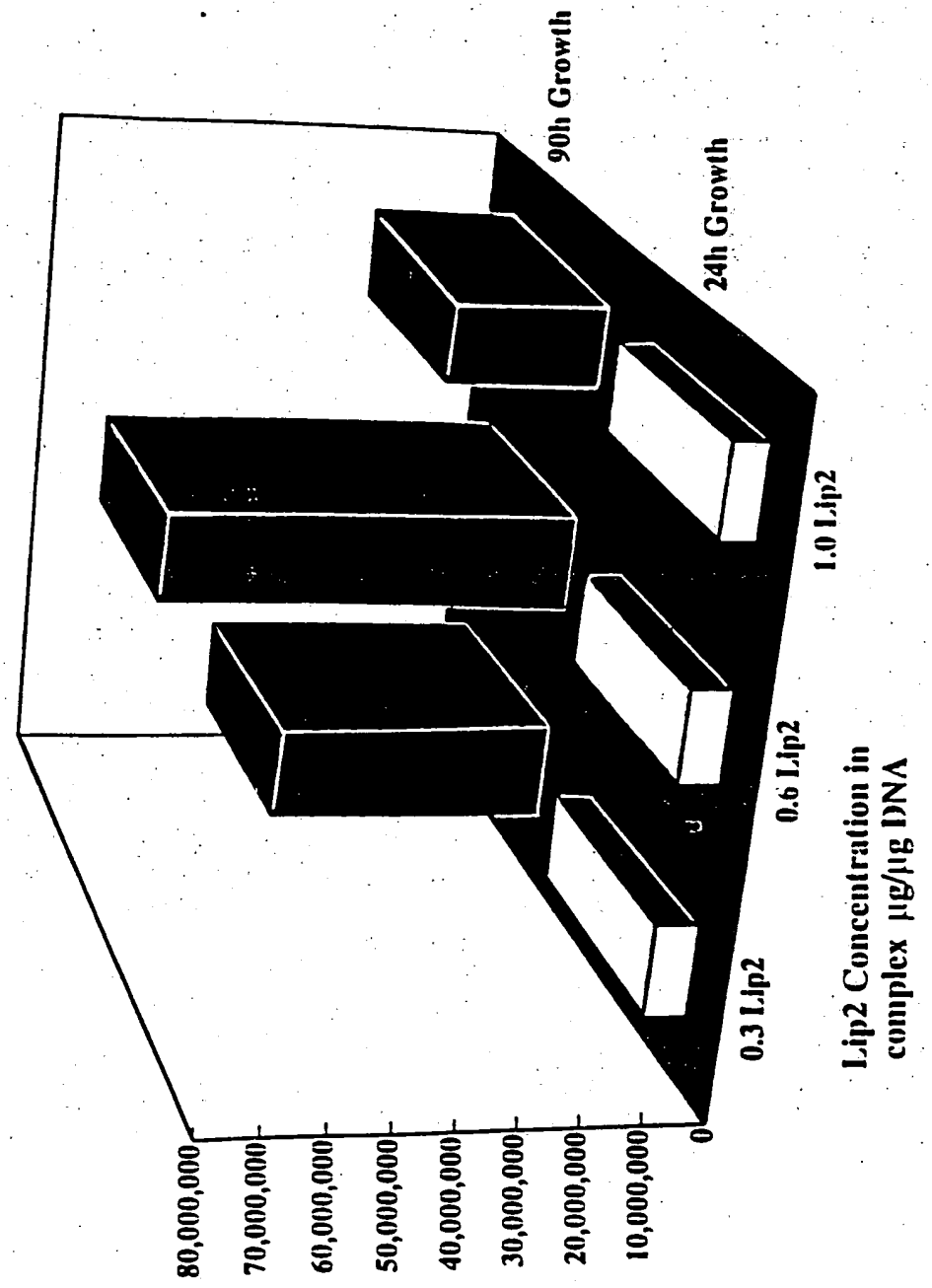

Non-targeted Delivery Vehicle
Insulin-NBC14
Insulin Concentration (µg/ml)
0
0.5
1
2.5
5
10
20
50
200
500
Luciferase Activity (RLU/mg protein)
0
200,000
400,000
600,000
800,000
1,000,000
1,200,000
1,400,000
1,600,000
1,800,000
2,000,000

FIG. 16


Luciferase Activity
(RLU/mg protein)
80,000,000
70,000,000
60,000,000
50,000,000
40,000,000
30,000,000
20,000,000
10,000,000
0
0.3 Ljp2
0.6 Ljp2
1.0 Ljp2
Ljp2 Concentration in complex µg/µg DNA
24h Growth
90h Growth

28/50

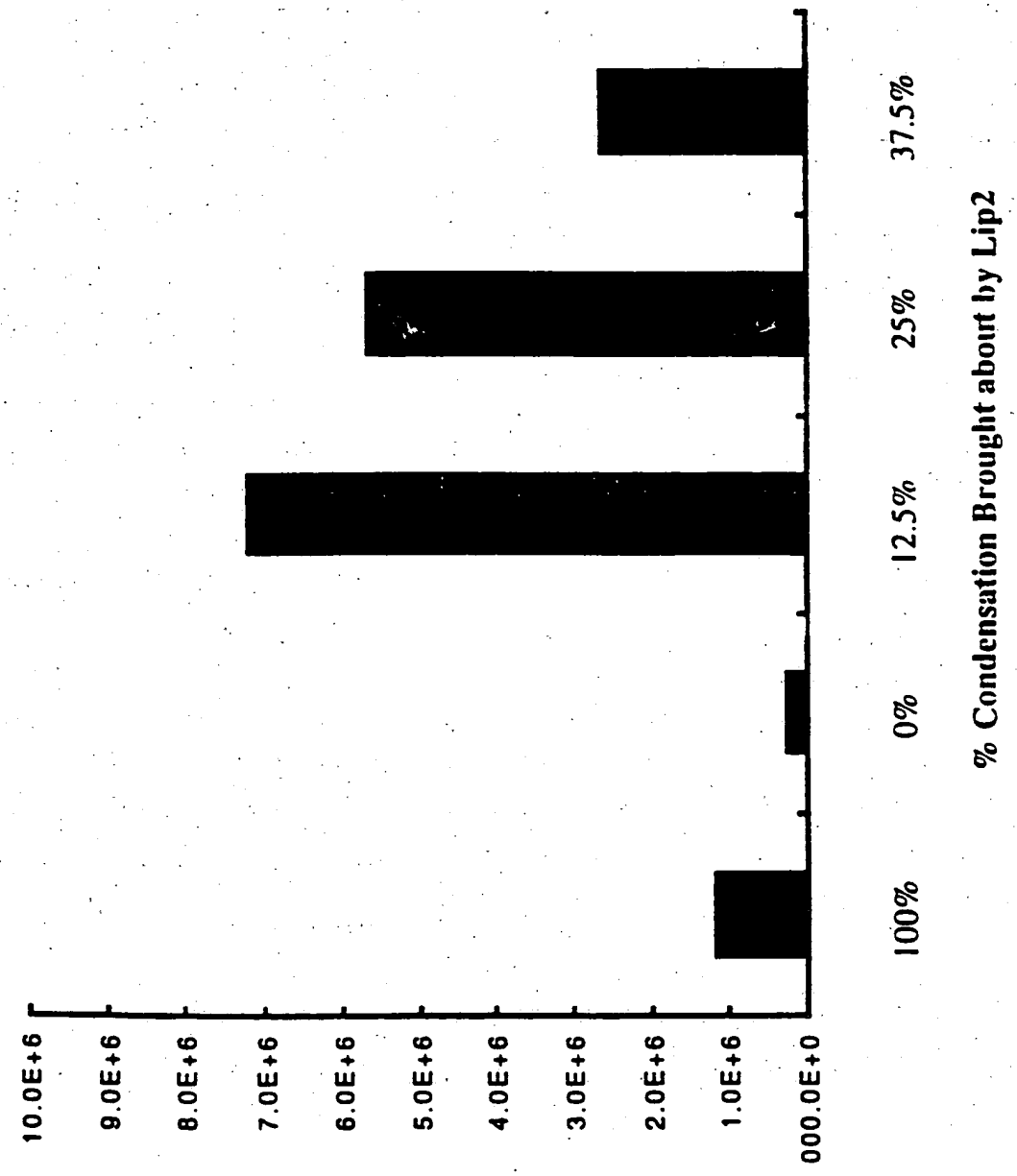

Luciferase Activity RLU/mg protein
10.0E+6
9.0E+6
8.0E+6
7.0E+6
6.0E+6
5.0E+6
4.0E+6
3.0E+6
2.0E+6
1.0E+6
000.0E+0
100%
0%
12.5%
25%
37.5%
% Condensation Brought about by Lip2

FIG.17

FIG. 18

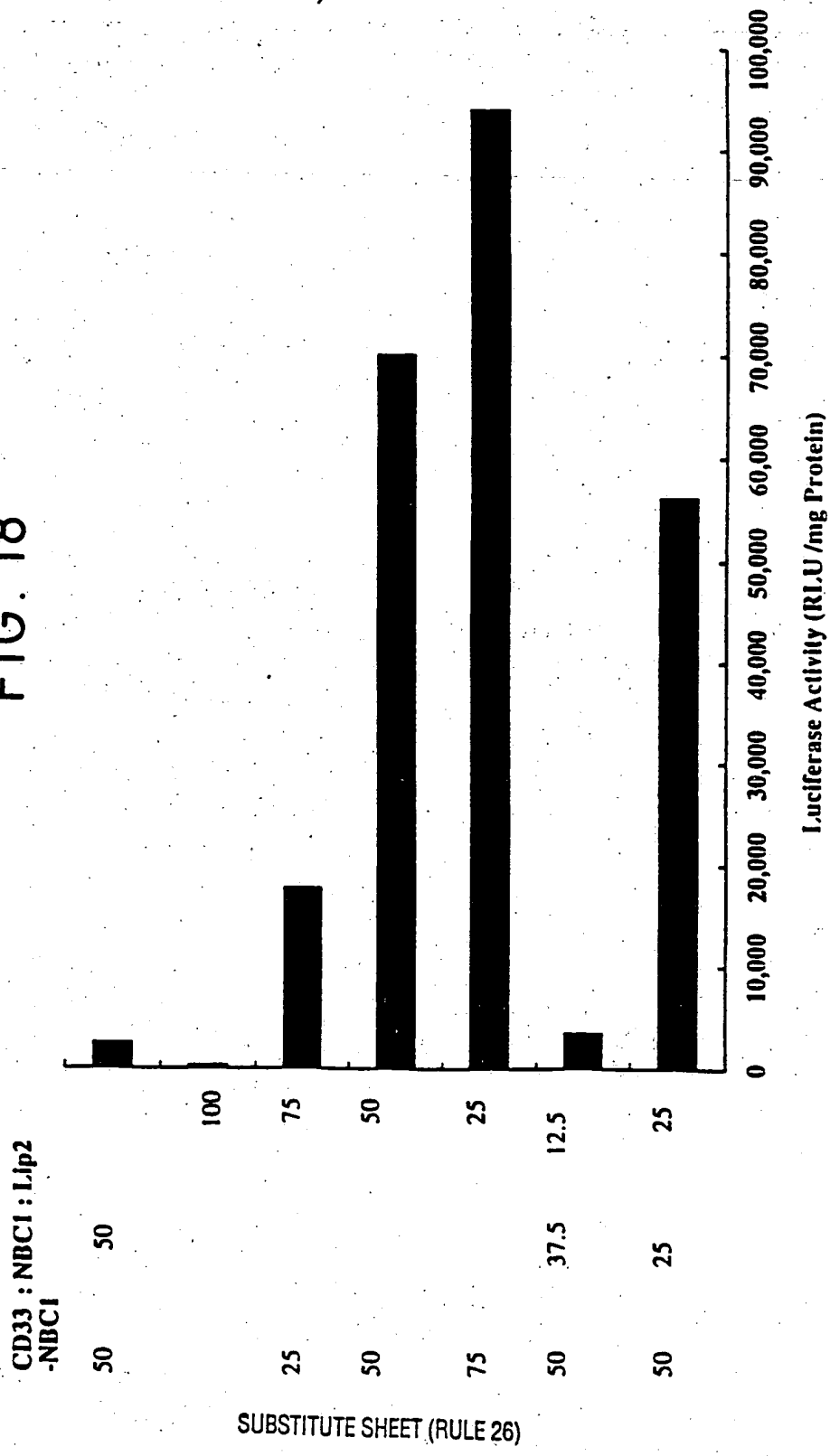
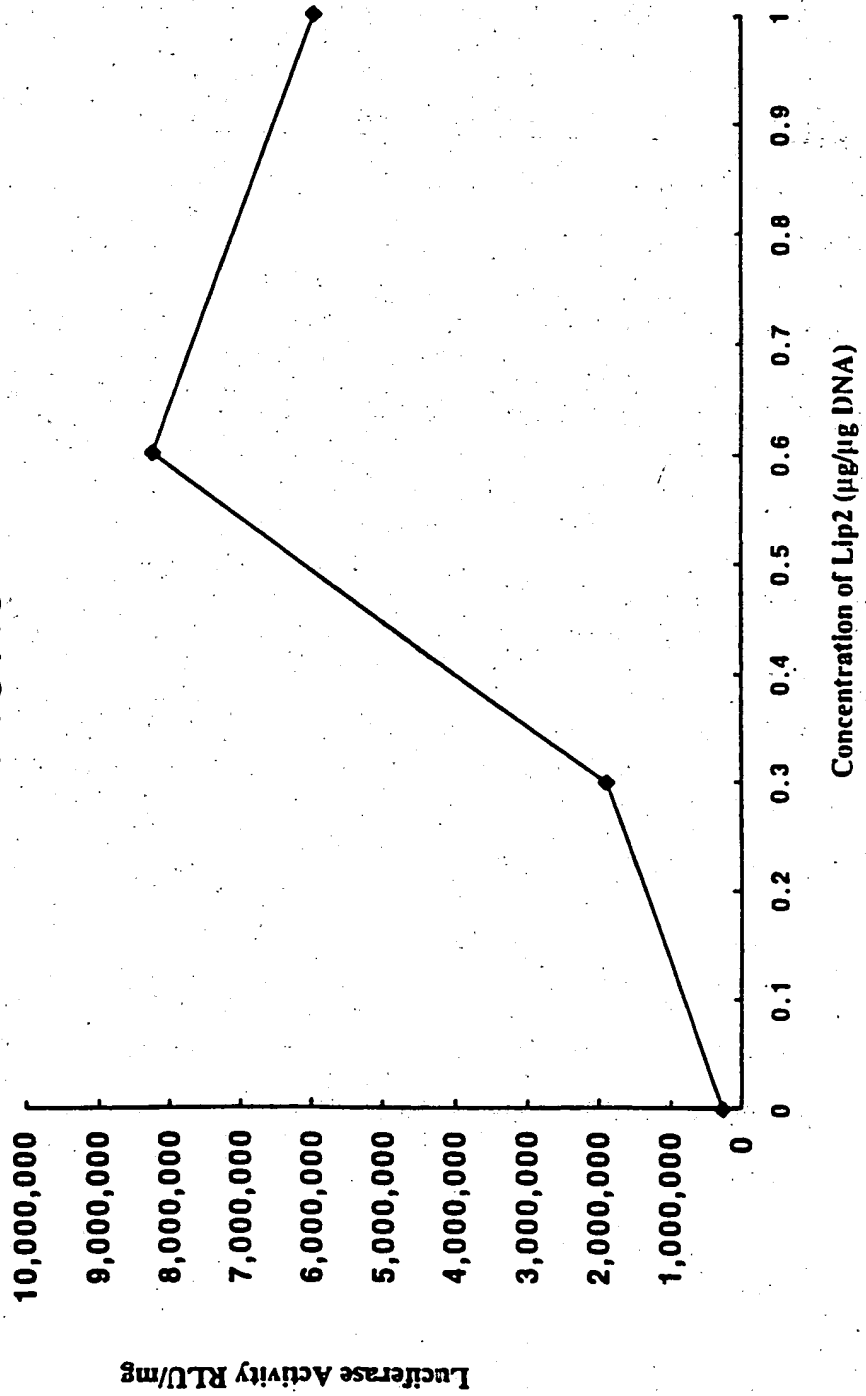

Luciferase Activity (RLU/mg Protein)
0
10,000
20,000
30,000
40,000
50,000
60,000
70,000
80,000
90,000
100,000
CD33 : NBC1 : Lip2
-NBC1
50 50
100
50 25
75
50 50
50
25
50 75
25
12.5
50 37.5
25
50 50
25

FIG. 19


Luciferase Activity RLU/mg
10,000,000
9,000,000
8,000,000
7,000,000
6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Concentration of Lip2 (µg/µg DNA)

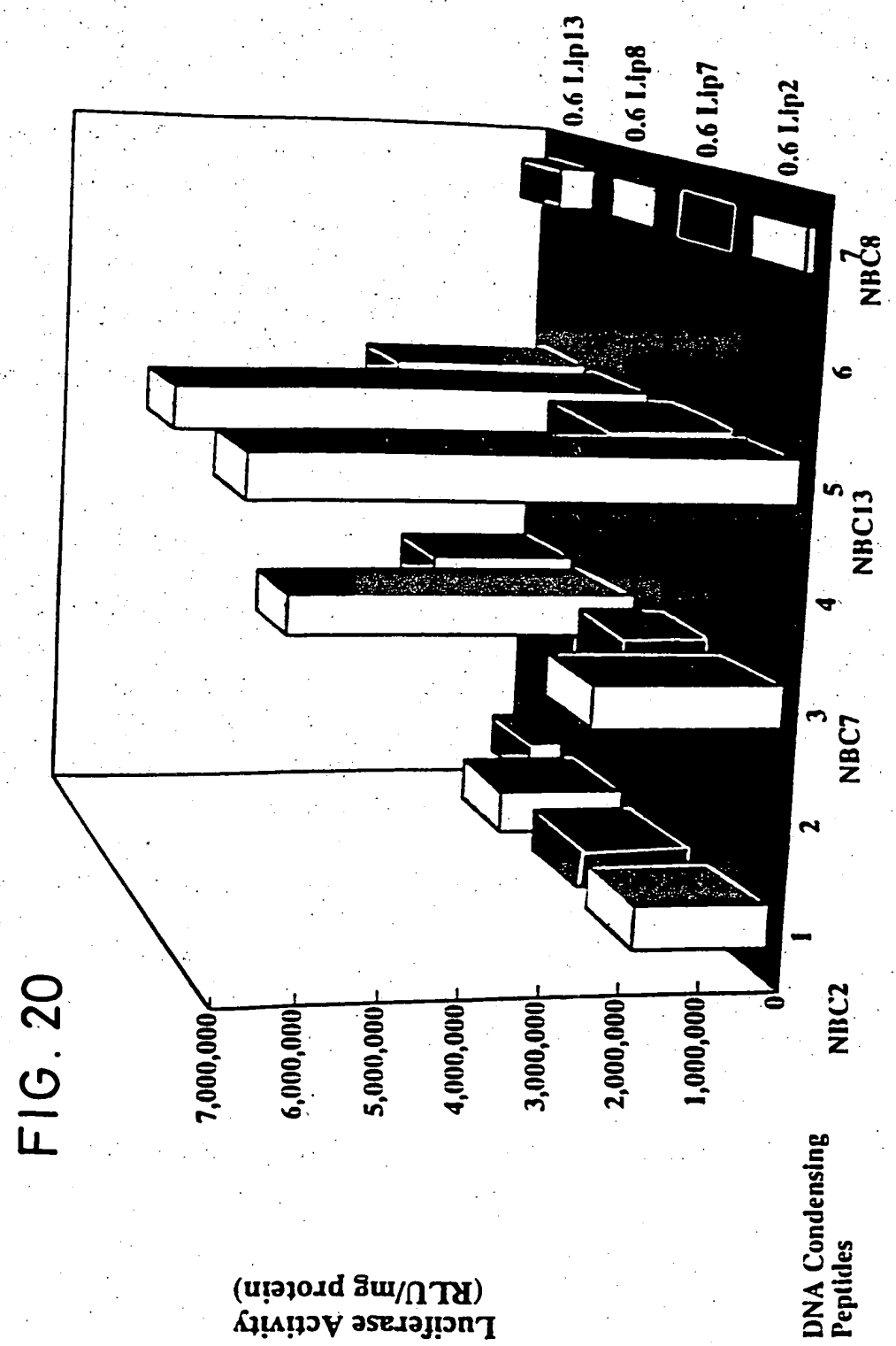

0.6 LIp13
0.6 LIp8
0.6 LIp7
0.6 LIp2
NBC8
NBC13
NBC7
NBC2
1
2
3
4
5
6
7
0
1,000,000
2,000,000
3,000,000
4,000,000
5,000,000
6,000,000
7,000,000
Luciferase Activity
(RLU/mg protein)
DNA Condensing
Peptides

FIG. 20

FIG. 21

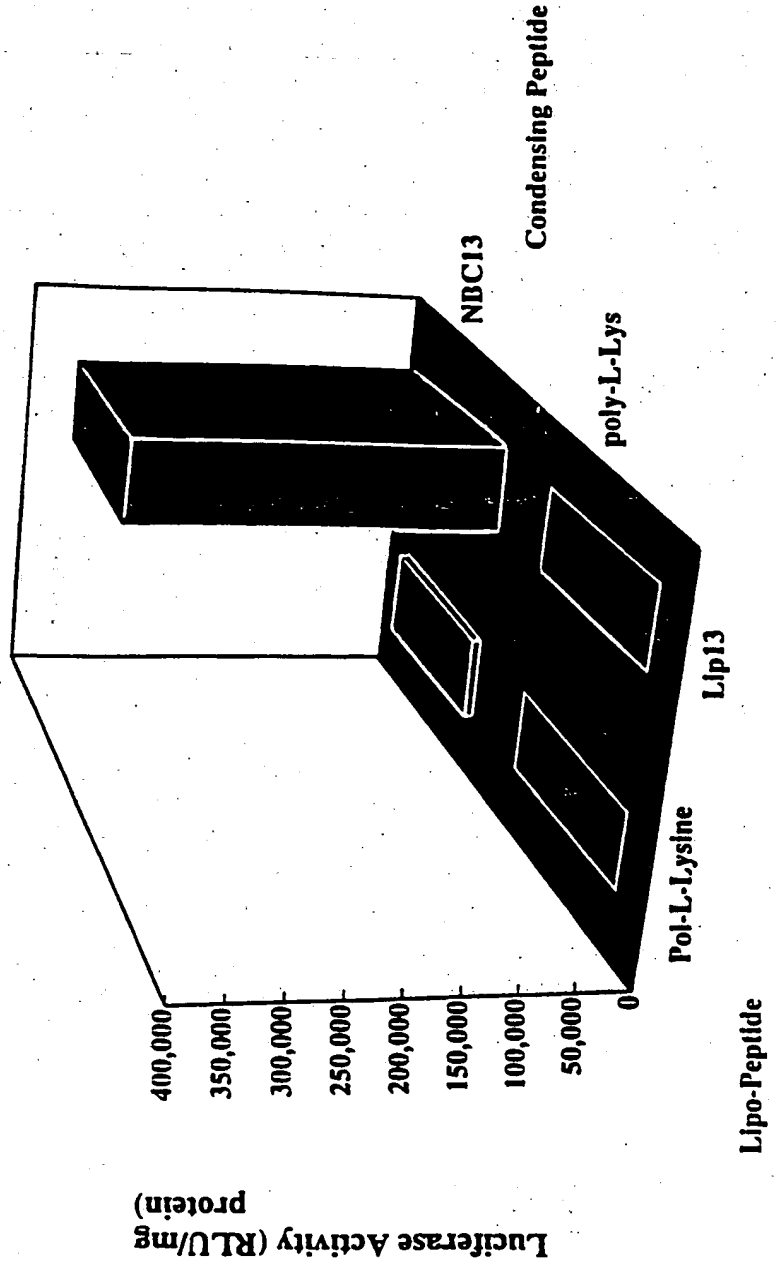
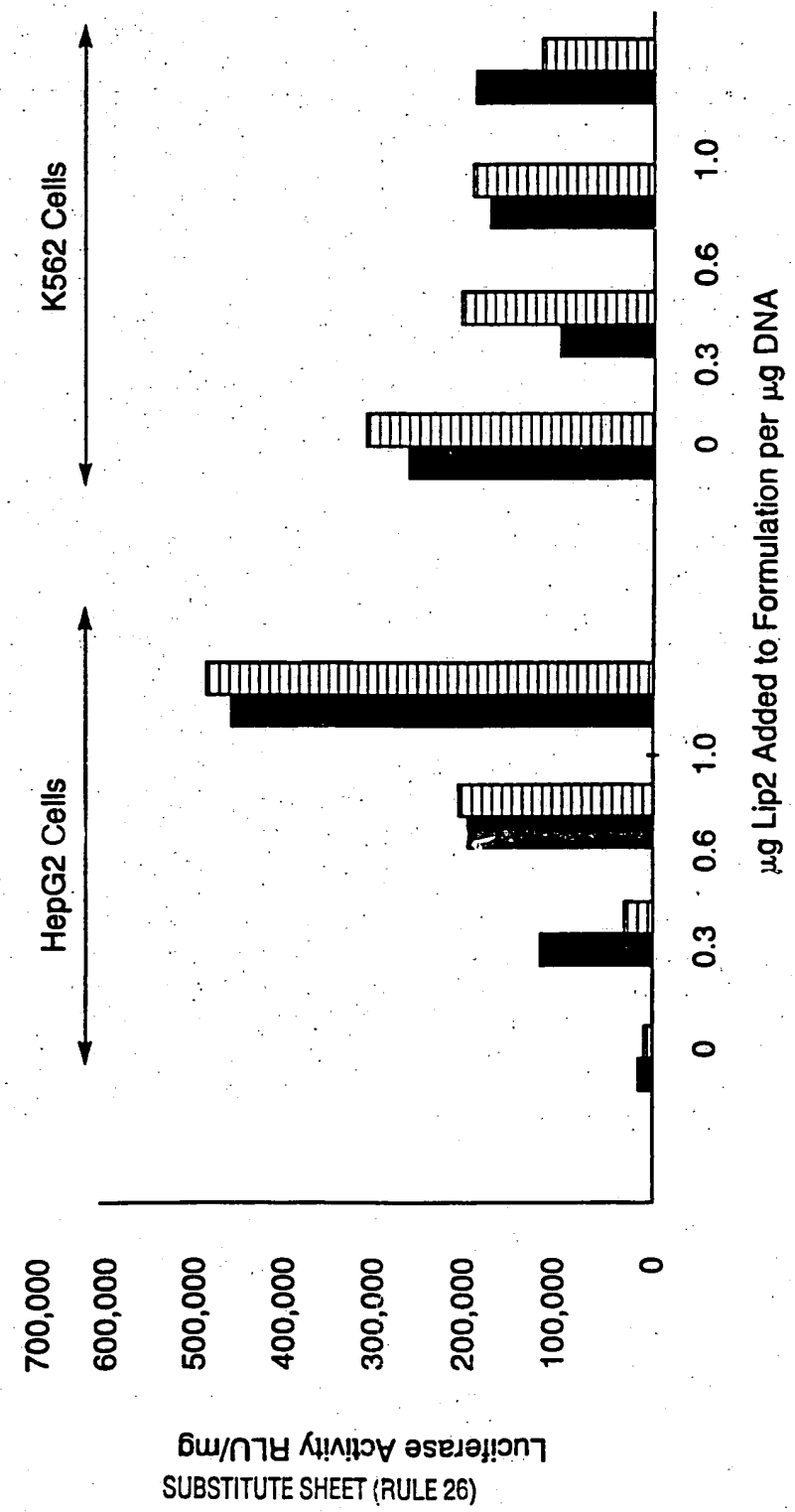

Condensing Peptide
NBC13
poly-L-Lys
Lip13
Pol-L-Lysine
Lipo-Peptide
0
50,000
100,000
150,000
200,000
250,000
300,000
350,000
400,000
Luciferase Activity (RLU/mg protein)

FIG. 22


K562 Cells
HepG2 Cells
0
0.3
0.6
1.0
0
0.3
0.6
1.0
µg Lip2 Added to Formulation per µg DNA
0
100,000
200,000
300,000
400,000
500,000
600,000
700,000
Luciferase Activity RLU/mg

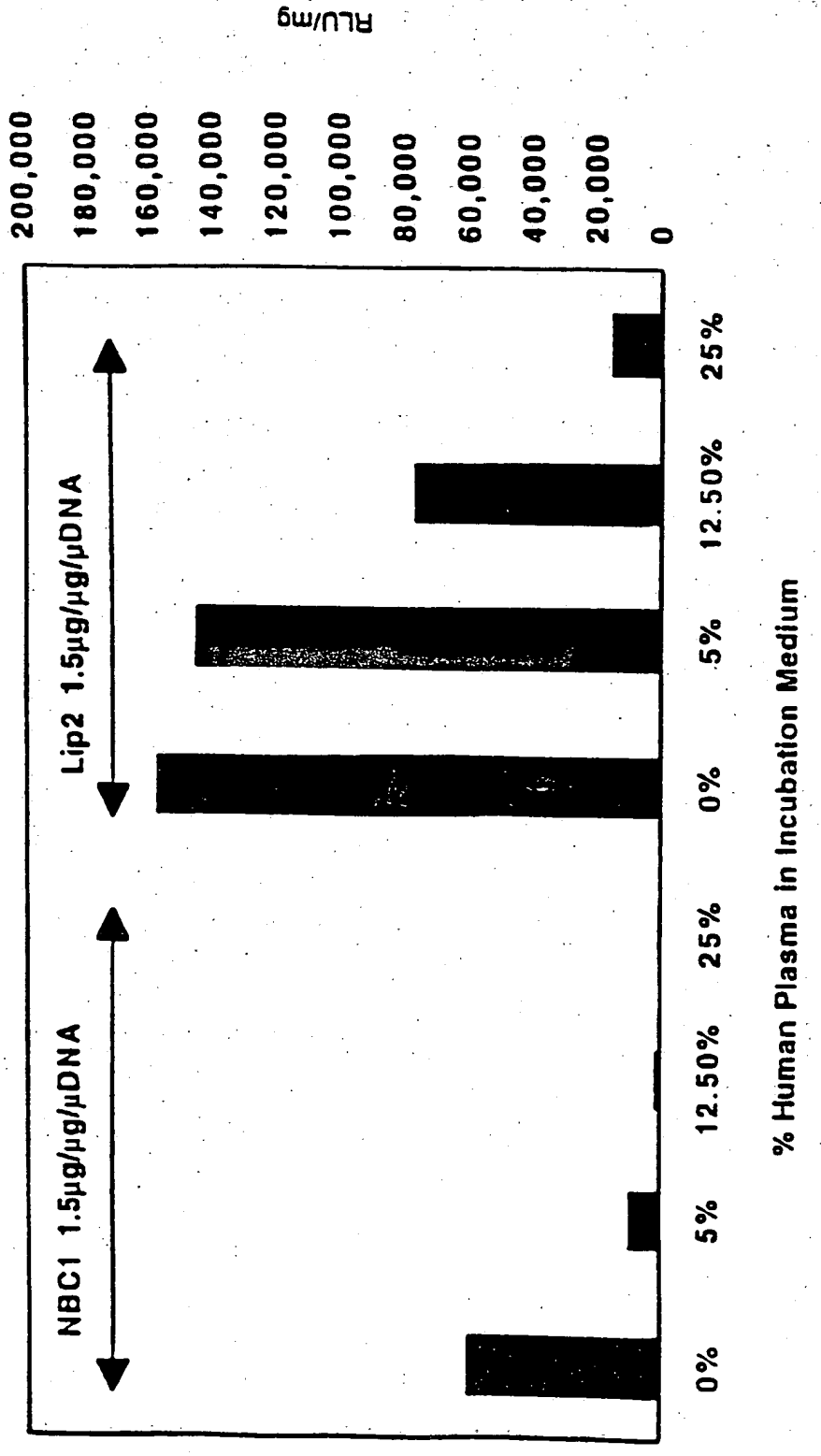

RLU/mg
200,000
180,000
160,000
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0
Lip2 1.5µg/µg/µDNA
NBC1 1.5µg/µg/µDNA
0%
5%
12.50%
25%
0%
5%
12.50%
25%
% Human Plasma In Incubation Medium

FIG. 23

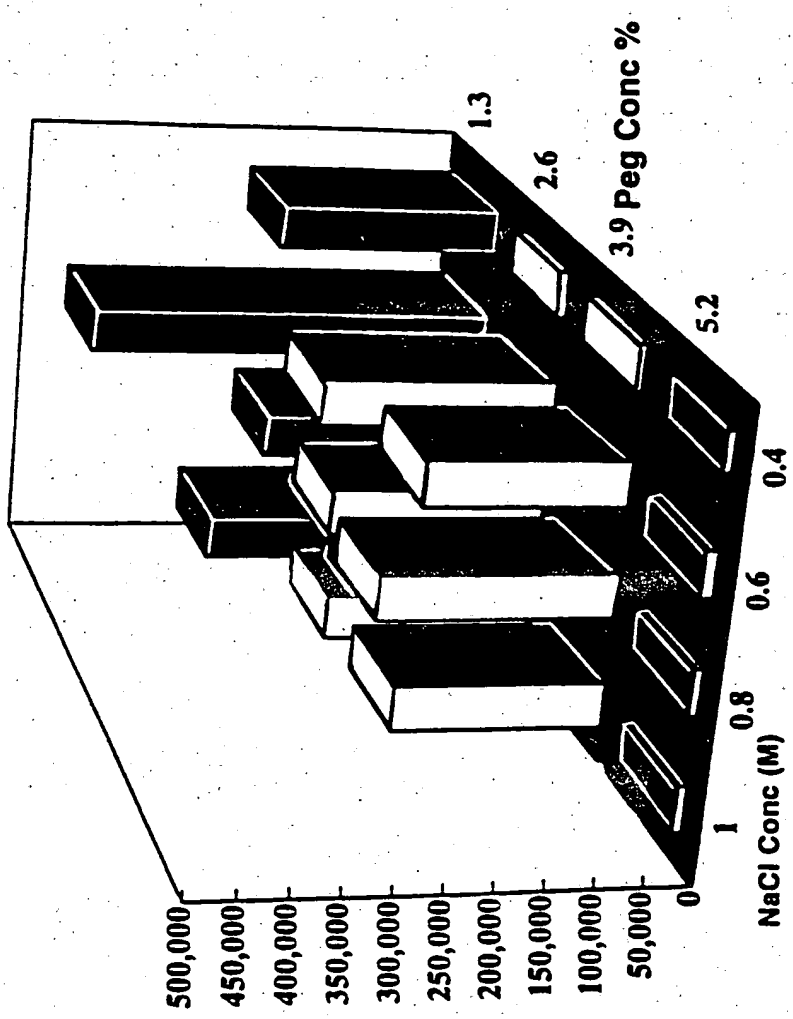

Luciferase Activity
(RLU/mg protein)
500,000
450,000
400,000
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
NaCl Conc (M)
1
0.8
0.6
0.4
3.9 Peg Conc %
1.3
2.6
5.2

FIG. 24

FIG. 25

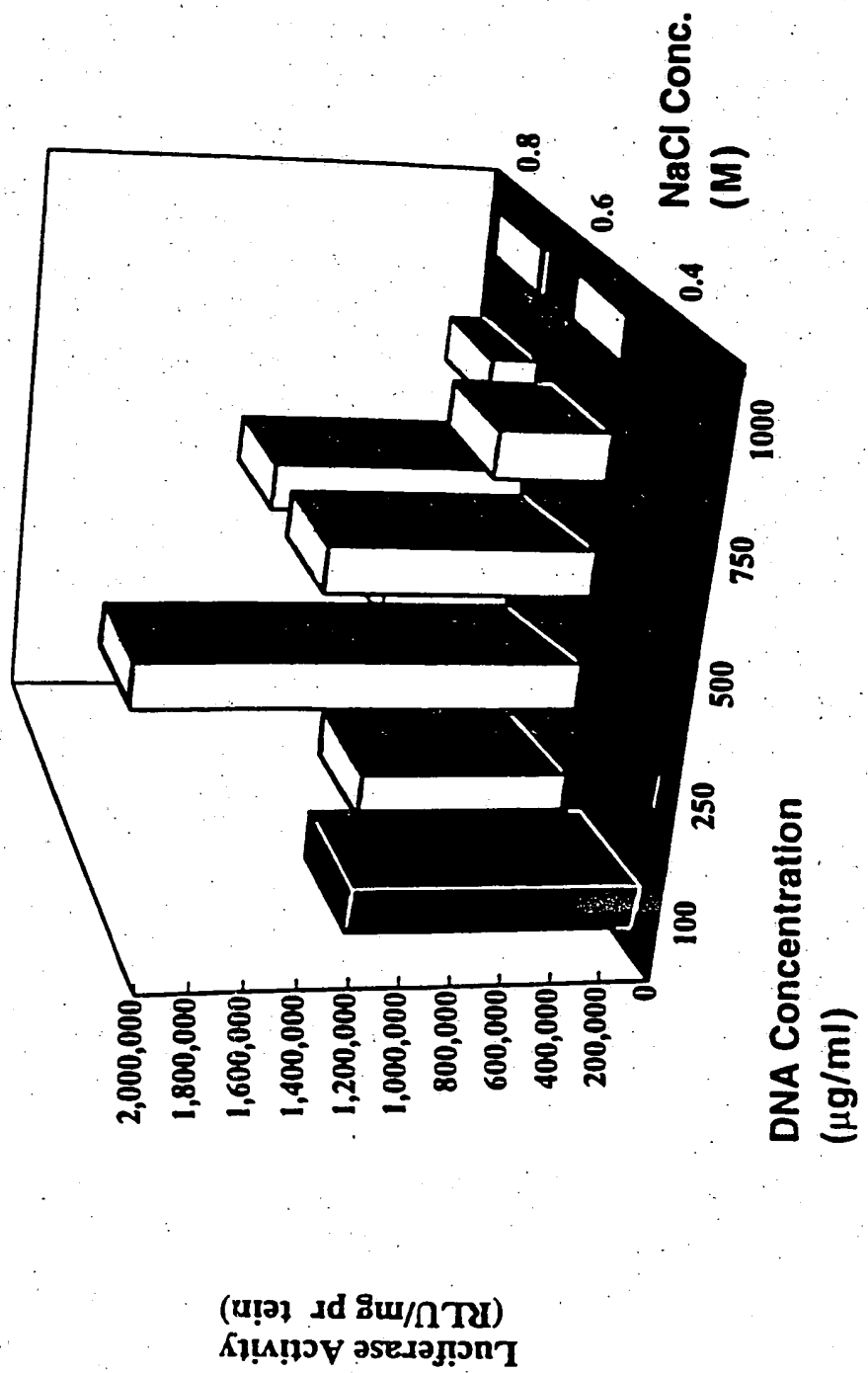

Luciferase Activity
(RLU/mg protein)
2,000,000
1,800,000
1,600,000
1,400,000
1,200,000
1,000,000
800,000
600,000
400,000
200,000
0
100
250
500
750
1000
DNA Concentration
(µg/ml)
0.4
0.6
0.8
NaCl Conc.
(M)

FIG. 26(a)

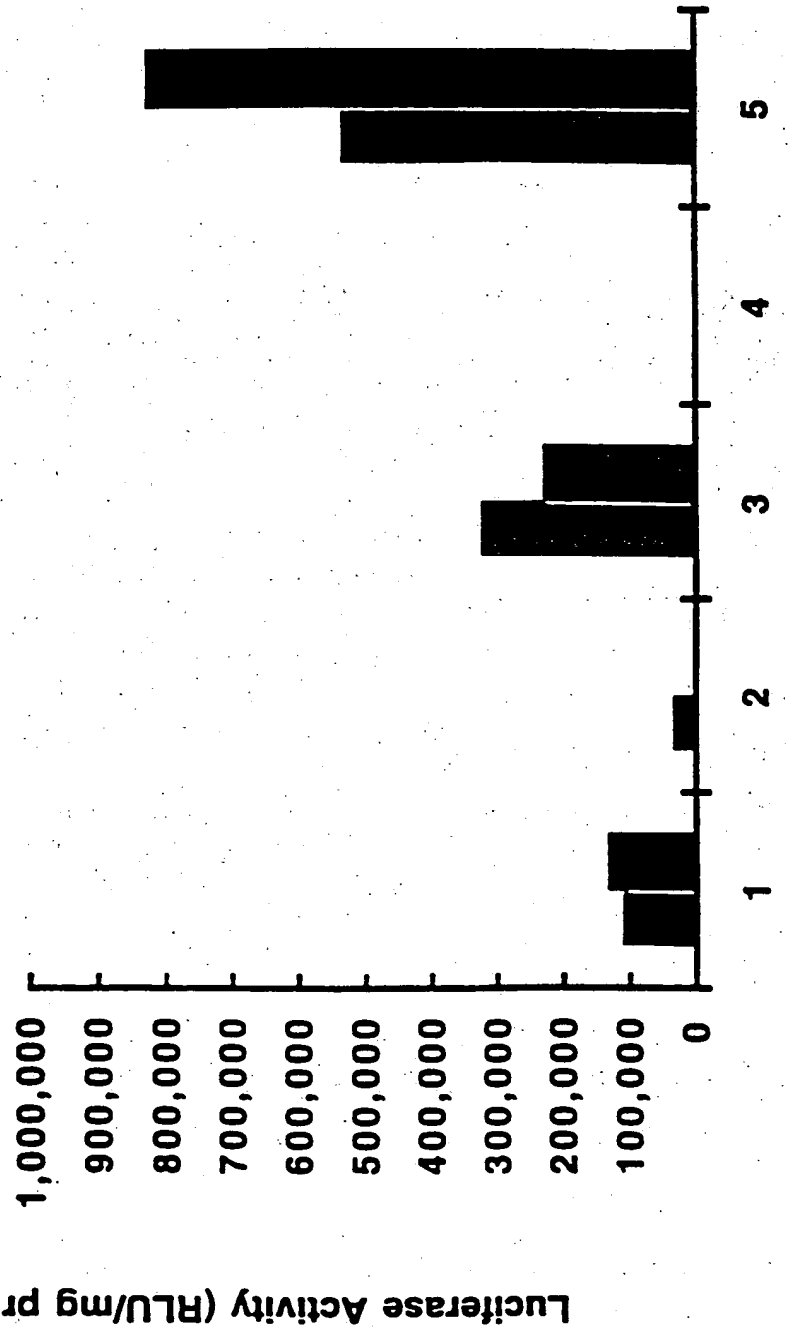

Luciferase Activity (RLU/mg protein)
1,000,000
900,000
800,000
700,000
600,000
500,000
400,000
300,000
200,000
100,000
0
1
2
3
4
5

FIG. 26(b)

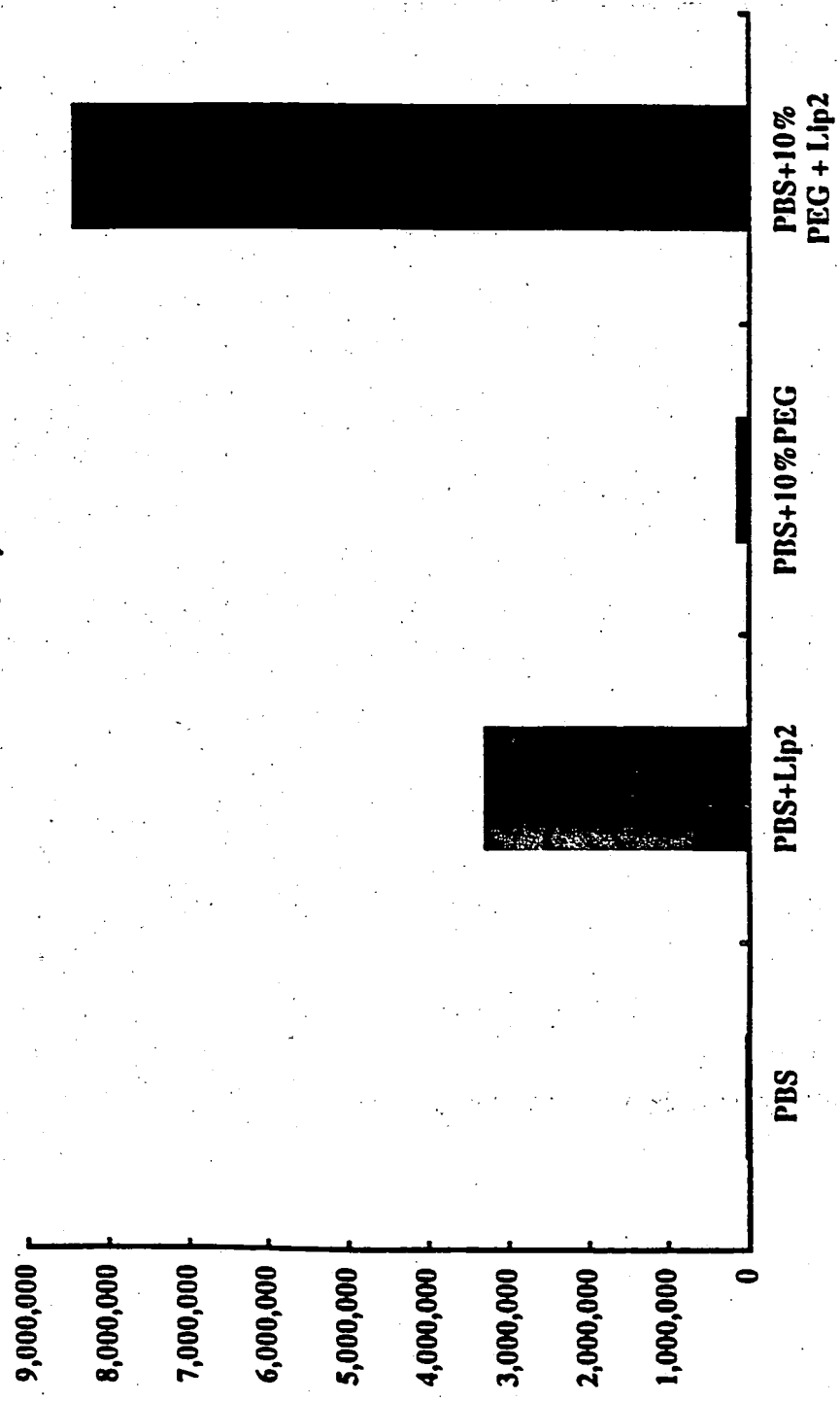

Luciferase Activity (RLU/mg protein)
9,000,000
8,000,000
7,000,000
6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
PBS
PBS+Lip2
PBS+10%PEG
PBS+10% PEG + Lip2

FIG. 27

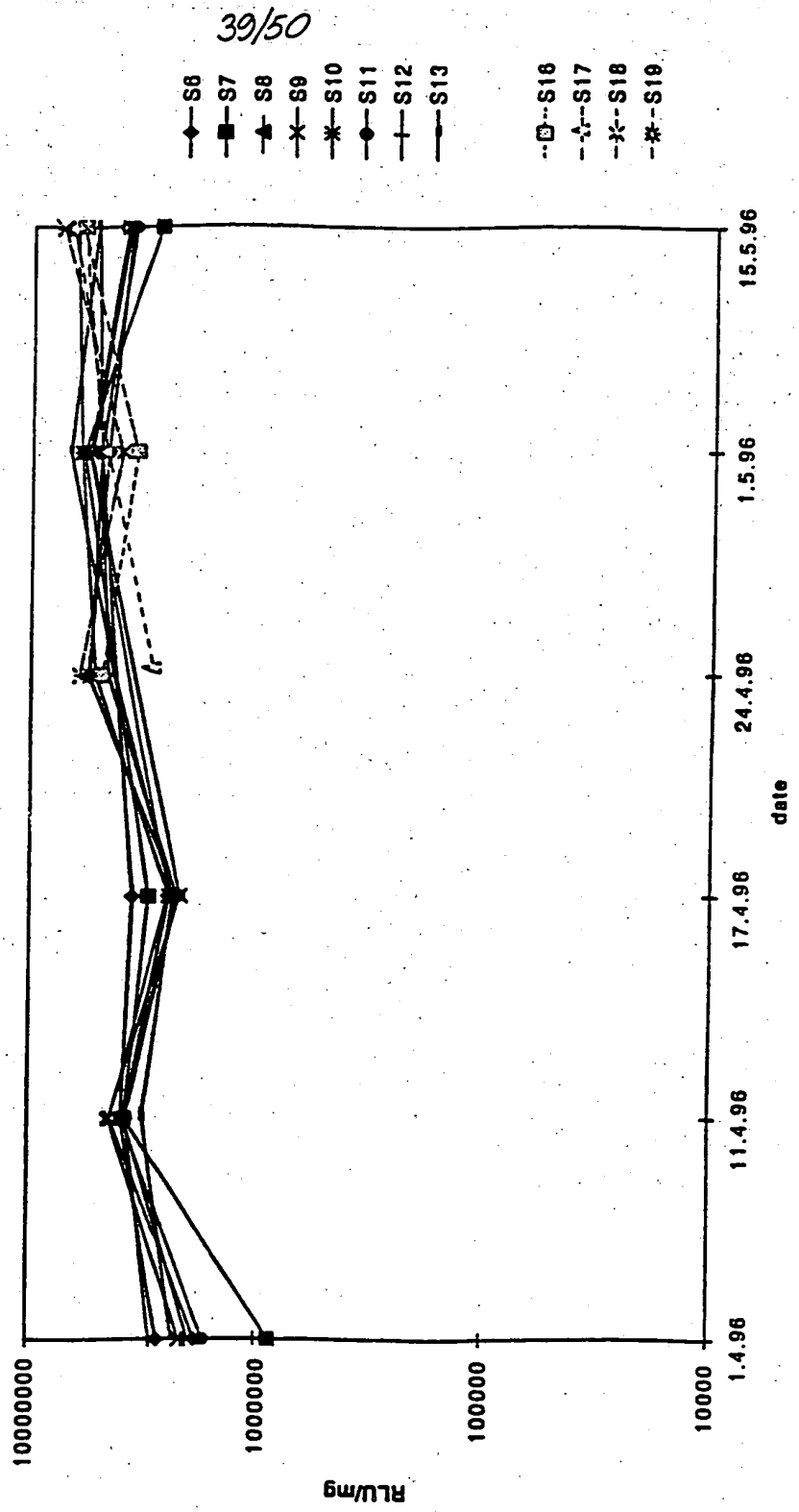
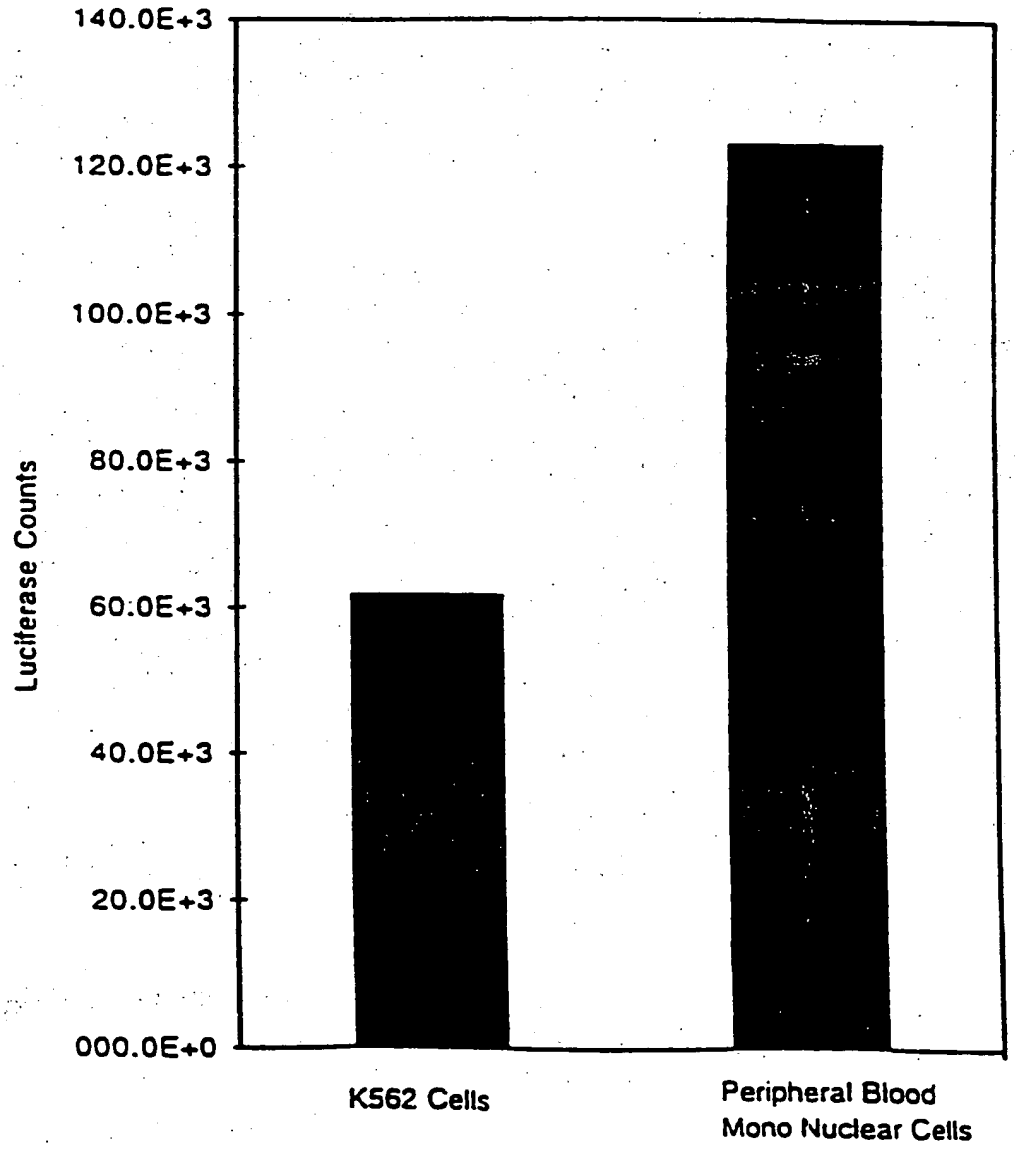

stability data
S6
S7
S8
S9
S10
S11
S12
S13
S16
S17
S18
S19
10000000
1000000
100000
10000
RLU/mg
1.4.96
11.4.96
17.4.96
24.4.96
1.5.96
15.5.96
date

FIG. 28


140.0E+3
120.0E+3
100.0E+3
80.0E+3
60.0E+3
40.0E+3
20.0E+3
000.0E+0
Luciferase Counts
K562 Cells
Peripheral Blood
Mono Nuclear Cells

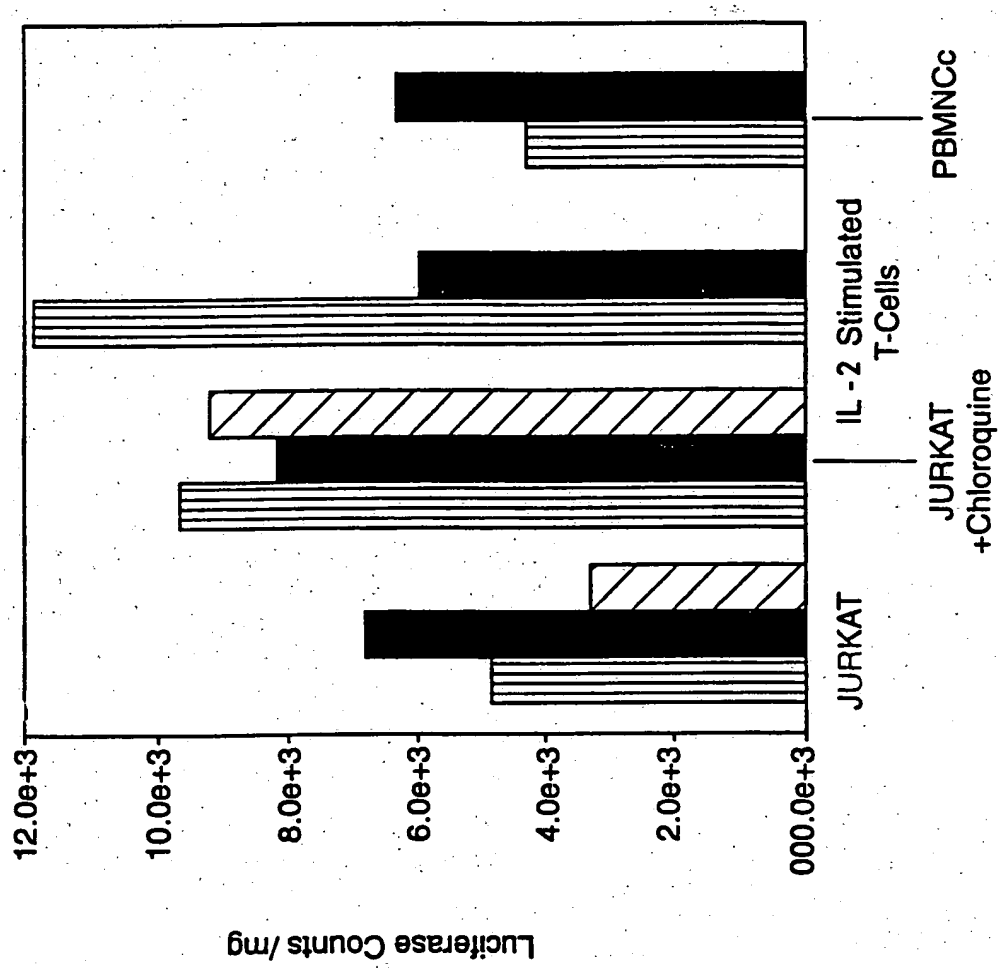
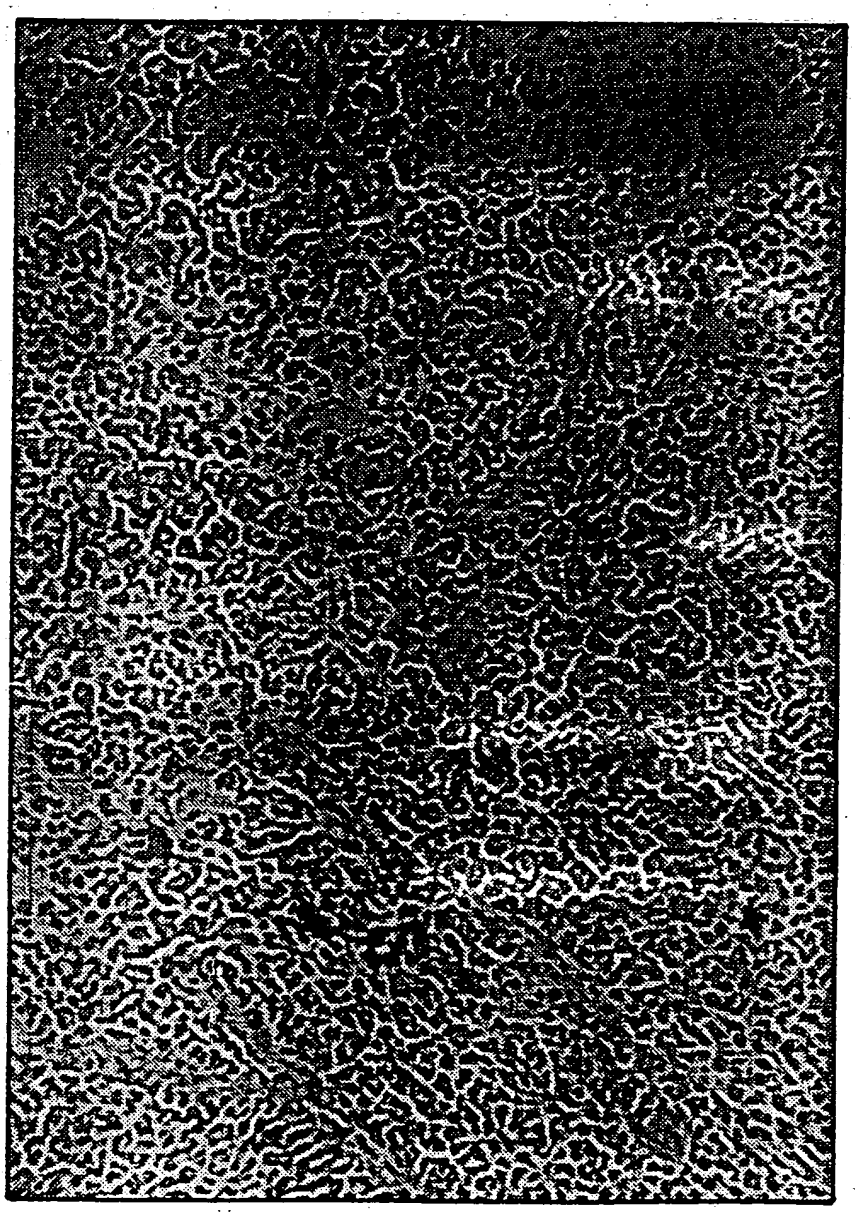
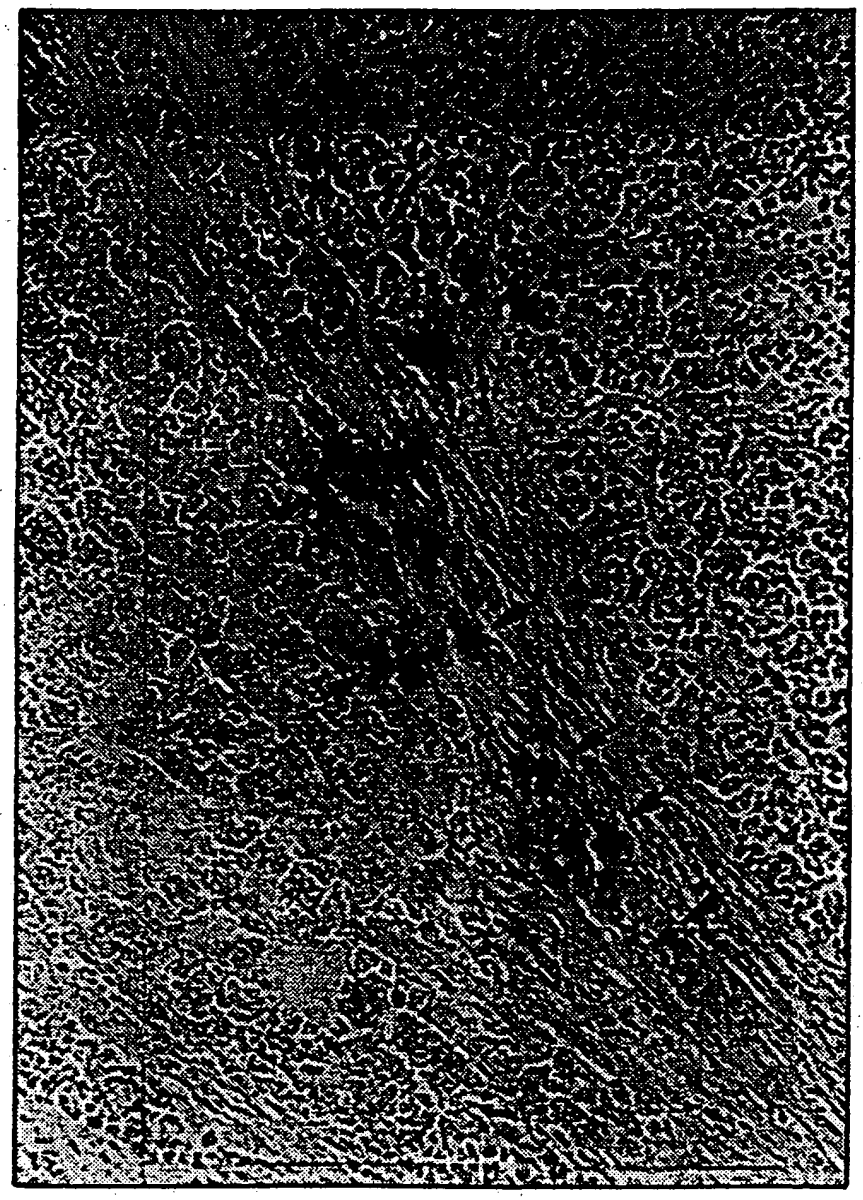

Luciferase Counts /mg
12.0e+3
10.0e+3
8.0e+3
6.0e+3
4.0e+3
2.0e+3
000.0e+3
JURKAT
JURKAT +Chloroquine
IL - 2 Stimulated T-Cells
PBMNCc

FIG. 29



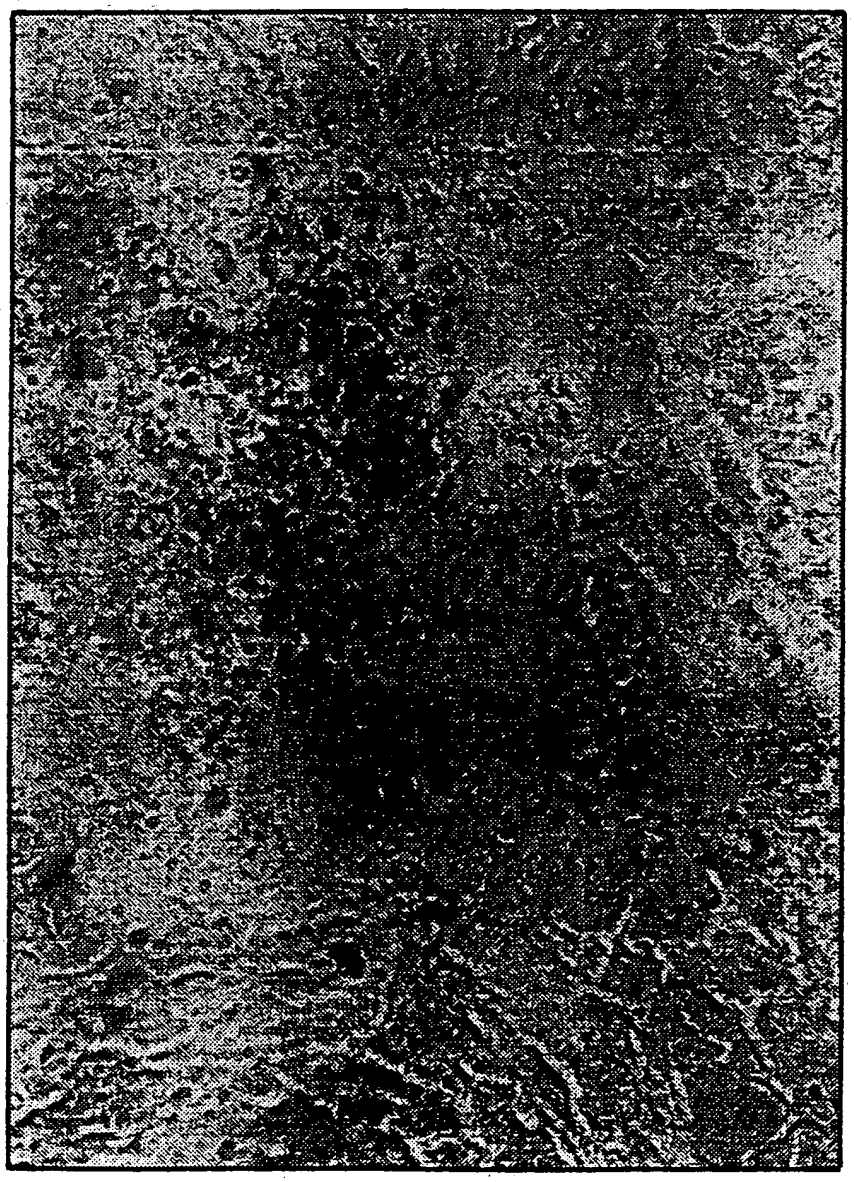
SLIDE 1 ANIMAL A

FIG. 30



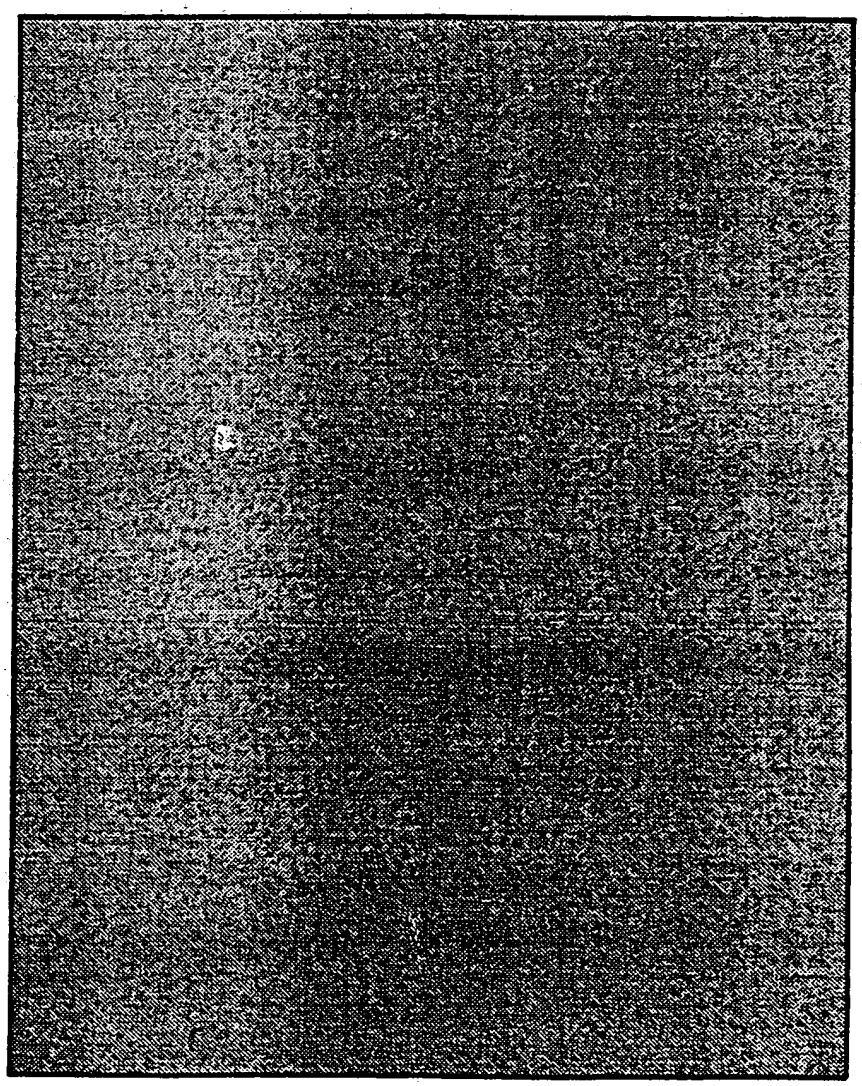
SLIDE 2 ANIMAL B

FIG. 30 (CONTD.)



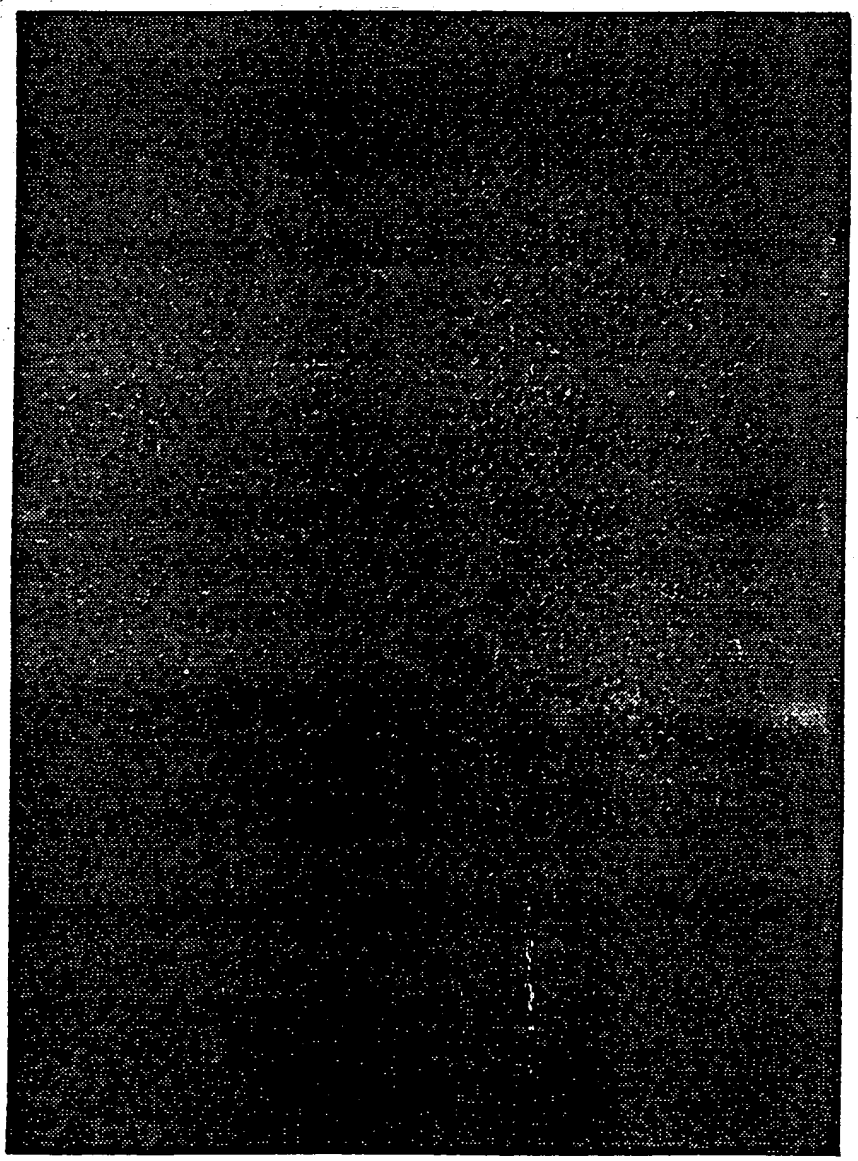
SLIDE 3 ANIMAL C

FIG. 30 (CONTD.)



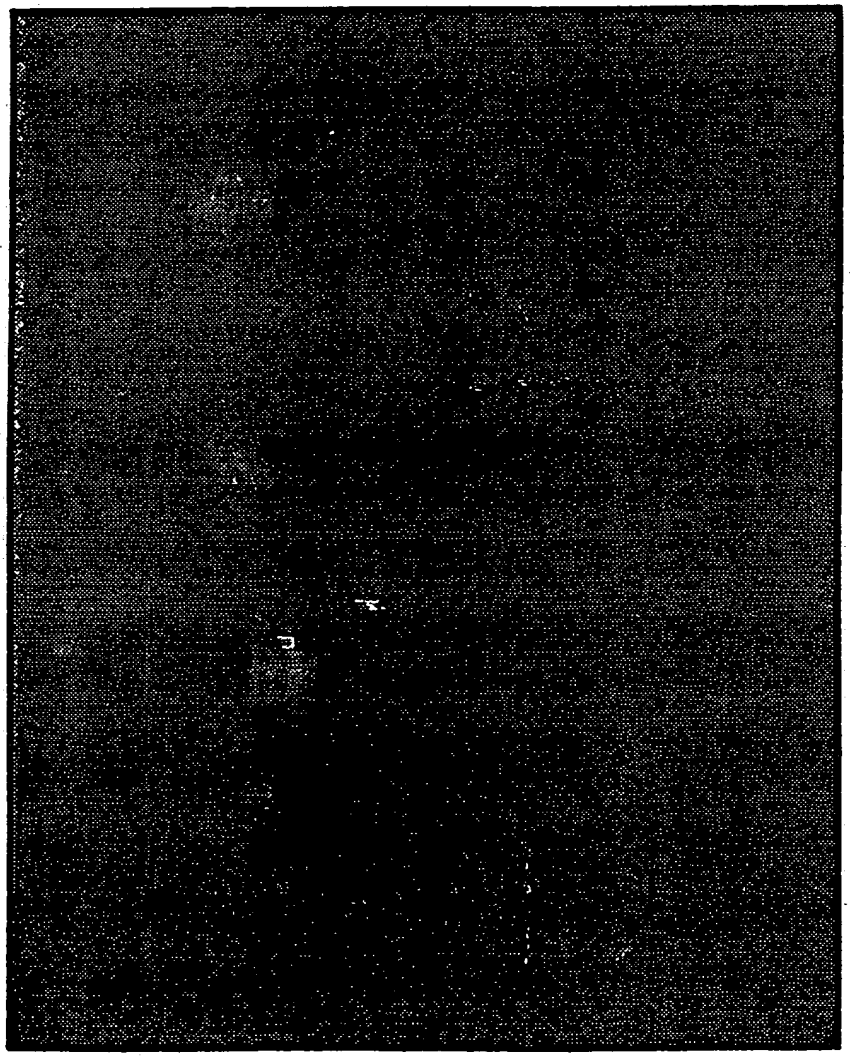
TUMOUR 1

FIG. 31(a)



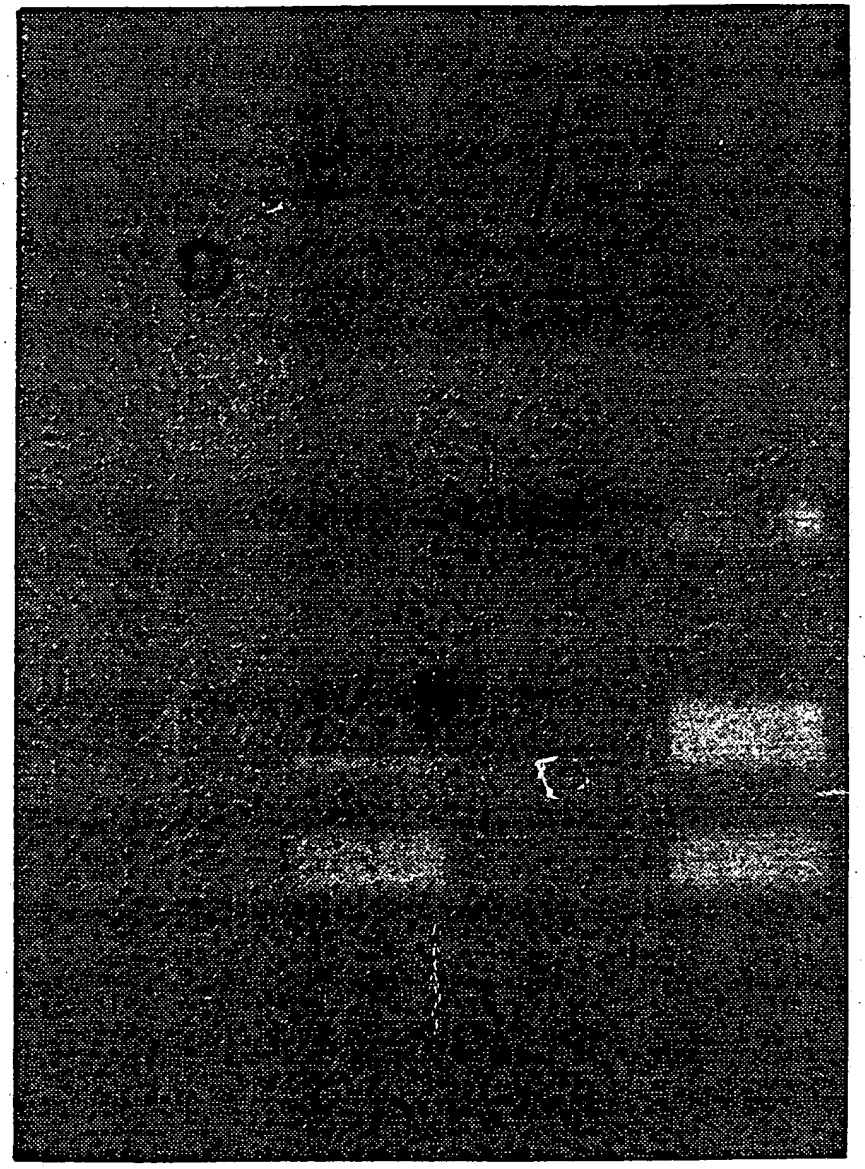
TUMOUR 2

FIG. 31(b)



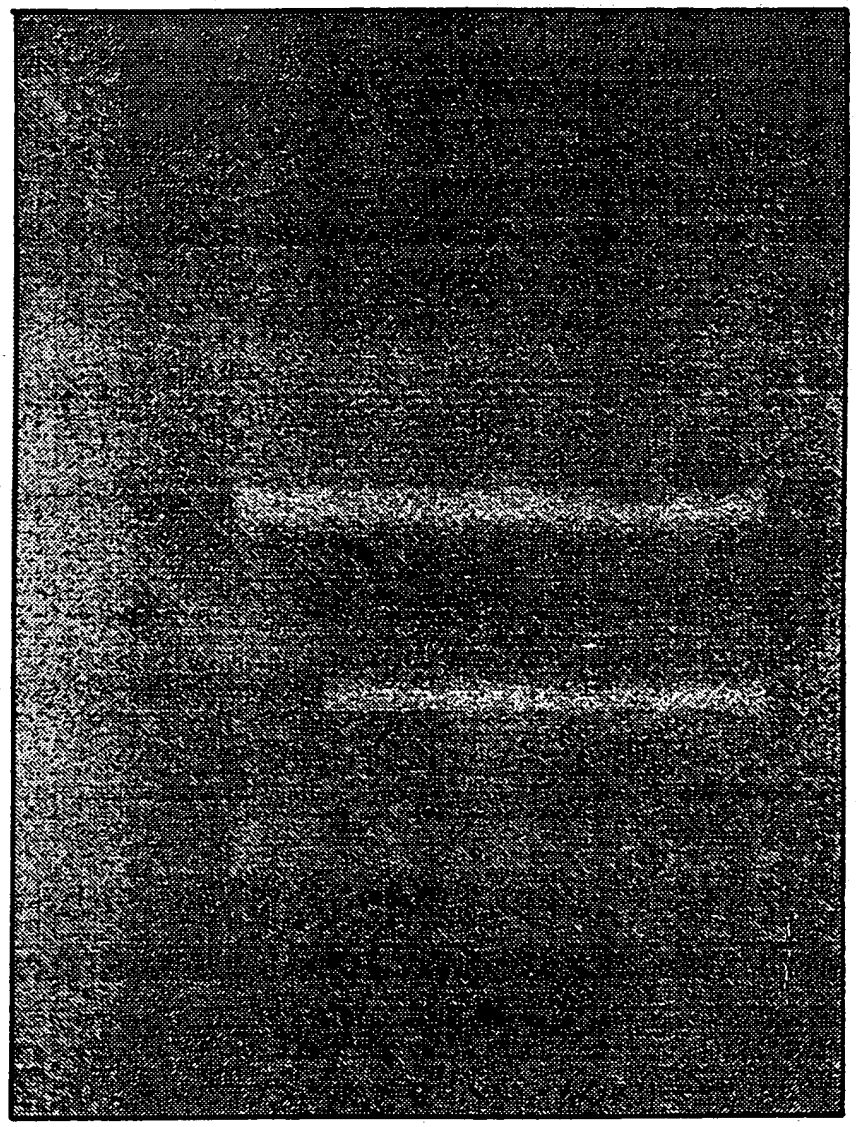
TUMOUR 3

FIG. 31(c)



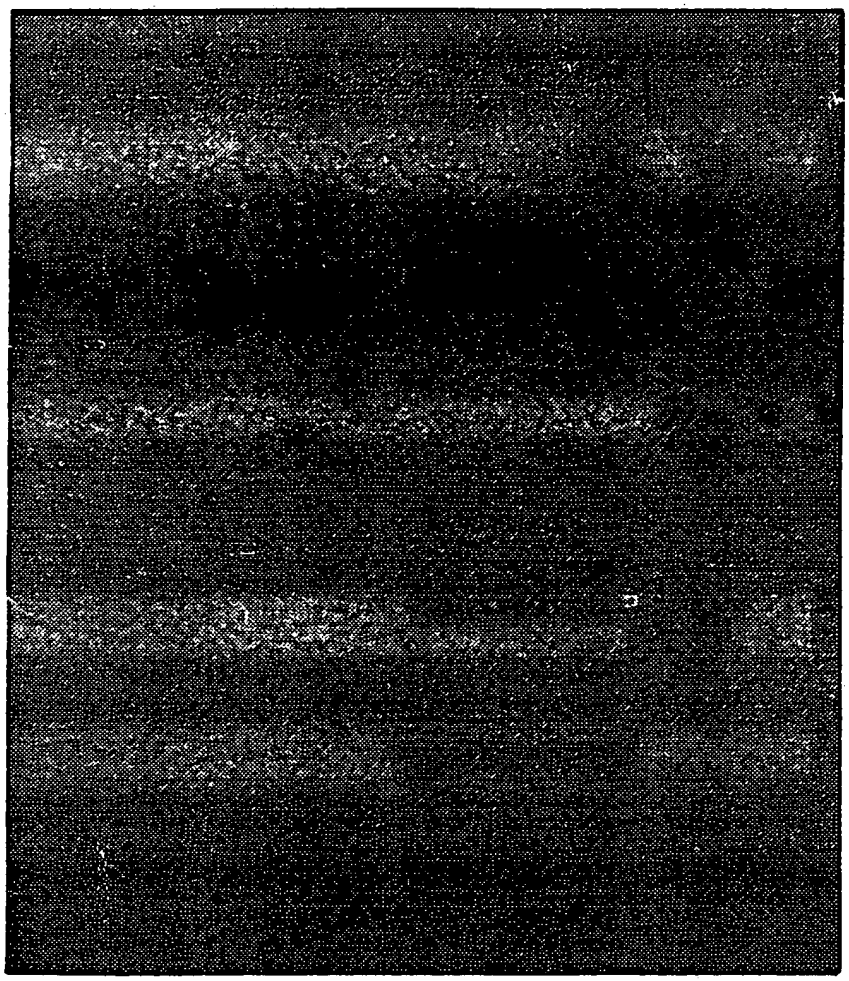
TUMOUR 4

FIG. 31(d)



TUMOUR 5

FIG. 31(e)



TUMOUR 6

FIG. 31(f)